The Pennsylvania State University
The Graduate School
Department of Chemistry

INVESTIGATING ROLES OF UNCONVENTIONAL RNA PRIMARY, SECONDARY, AND TERTIARY MOTIFS IN REGULATION OF THE PROTEIN KINASE PKR

A Dissertation in
Chemistry
by
Rebecca Toroney

© 2010 Rebecca Toroney

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

December 2010
The dissertation of Rebecca Toroney was reviewed and approved* by the following:

Philip C. Bevilacqua  
Professor of Chemistry  
Dissertation Advisor  
Chair of Committee

Nicholas Winograd  
Evan Pugh Professor of Chemistry

Scott Showalter  
Assistant Professor of Chemistry

Craig E. Cameron  
Paul Berg Professor of Biochemistry and Molecular Biology

Barbara J. Garrison  
Shapiro Professor of Chemistry  
Head of the Department of Chemistry

*Signatures are on file in the Graduate School
ABSTRACT

Much is known about the importance of protein folding and structure in human health. Like protein, RNA is a biopolymer essential to cellular function that is known to adopt multiple alternative structures, yet much less is known about the possible involvement of RNA folding in human disease. The protein kinase PKR is a component of the innate immune response in humans that functions by sensing molecular patterns in RNA, specifically long stretches of double-stranded RNA (dsRNA) that accumulate in the cell during viral infection. Activation of PKR ultimately leads to the shut down of cellular translation and thus viral proliferation. In this way, the ability of PKR to recognize and be activated by a wide variety of RNA structural elements is crucial to maintaining the first line of defense against viral infection. The objective of this thesis is to characterize the structural basis of PKR activation and inhibition by various RNA elements at all three levels of the RNA folding hierarchy: primary, secondary, and tertiary. This research may ultimately provide a connection between RNA structure and human illness.

At the primary structural level, experiments were performed to establish the RNA structural determinants for PKR activation by single-stranded RNAs (ssRNA) with an essential triphosphate at the 5’-end. The requirement of 5’-triphosphate, which is not present in cellular RNA, for PKR activation by these ssRNAs is a novel discovery and was shown to be reproducible in vitro and in human cells. Activation assays, structure mapping techniques, and binding studies were performed which established that in addition to the dependency on triphosphate—and not diphosphate, monophosphate, or 7’-
methylguanosine, at the 5'-end—PKR activation by these ssRNAs requires short, ~5 bp stem loops located optimally near the center of the RNA. Additional experiments were conducted to characterize the binding pocket of this 5'-triphosphate in PKR. Competitive PKR activation assays and a variety of biophysical techniques including fluorescence competition, isothermal titration calorimetry (ITC), and fluorescence stopped-flow indicated that PKR binds the 5'-triphosphates of RNA at a site that is unique from the catalytic ATP binding site. Results from these studies also demonstrated that the 5'-triphosphate binding site is weaker in affinity and lower in specificity than the catalytic site, which potentially increases the ability of PKR to recognize a wider variety of pathogenic RNA 5'-end signatures.

At the secondary and tertiary structural levels, PKR regulation by various elements of hepatitis C virus (HCV IRES) RNA was investigated. Conflicting reports in the literature were resolved by performing activation and inhibition assays of the full-length IRES at a range of RNA concentrations, which established the role of the full-length HCV IRES as both an activator and inhibitor of PKR. Additionally, individual elements of the IRES secondary structure were identified as activators, with particular emphasis on domain II, which functions as a potent activator despite its limited number of canonical basepairs. Footprinting and mutational analyses as well as modeling were utilized to establish that noncanonical regions within domain II are critical for activation through mimicry of dsRNA structure. The role of IRES tertiary structure was also investigated through analysis of PKR activation by mutant IRES RNAs that lack this tertiary structure. Results obtained suggest that while tertiary structure promotes optimal PKR activation, the IRES still activates well in its absence.
Finally, a focused examination of the role of one specific RNA secondary motif, the tandem GA mismatch, was conducted. Tandem GA mismatches adopt one of two distinct conformations with disparate representation in biological RNA. The ability of PKR to distinguish between these two conformers was investigated through activation and inhibition assays of various model RNAs containing one or more mismatches of each tandem mismatch conformer. Experiments indicated a slight preference for PKR regulation by the biologically rare conformer, suggesting that small changes in RNA structure may serve as recognition motifs of foreign RNA for PKR.
# TABLE OF CONTENTS

LIST OF FIGURES ............................................................................................................. x

LIST OF TABLES .................................................................................................................. xiv

ABBREVIATIONS ................................................................................................................. xv

ACKNOWLEDGEMENTS ....................................................................................................... xvii

Chapter 1  Introduction ........................................................................................................ 1

1.1 Hierarchy of RNA Folding and Structure ................................................................. 1

1.2 Function and Structural Biology of PKR ................................................................. 5

1.3 RNA Structure-based Regulation of PKR ............................................................... 10

   1.3.1 dsRBM-RNA binding characteristics ............................................................. 10

   1.3.2 Primary structure-based regulation ................................................................. 11

   1.3.3 Secondary structure-based regulation ............................................................ 13

   1.3.4 Tertiary structure-based regulation ............................................................... 18

1.4 Thesis Objectives ........................................................................................................ 20

1.5 References ............................................................................................................... 23

Chapter 2  Triphosphate-dependent activation of PKR by RNAs with short stem-loops .... 30

2.1 Abstract ...................................................................................................................... 30

2.2 Introduction ............................................................................................................... 31

2.3 Materials and Methods ............................................................................................ 33

   2.3.1 Protein preparation ......................................................................................... 33

   2.3.2 RNA sequences ............................................................................................ 33

   2.3.3 Preparation, purification, and CIP treatment of RNA .................................. 36

   2.3.4 PKR activation assays ................................................................................... 38

   2.3.5 Calculation of RNA-dependence for activation (RNA dependency factor) ........ 39

   2.3.6 Calculation of 5′-triphosphate dependence for activation ......................... 40

   2.3.7 UV melting and mfold prediction .................................................................. 41

   2.3.8 Enzymatic structure mapping of RNA ......................................................... 41

   2.3.9 Fluorescence polarization analysis of binding ............................................. 42

   2.3.10 Cells ............................................................................................................. 43

   2.3.11 Transfection .................................................................................................. 44

   2.3.12 Western blotting ......................................................................................... 45

2.4 Results and discussion ............................................................................................... 45

   2.4.1 Activation of PKR by ss-dsRNA is 5′-triphosphate-dependent, while PKR activation by dsRNA is not ........................................... 45
2.4.2 Characterization of 1° and 2° structural elements in 5’-triphosphate-dependent activation of PKR by ssRNA

*in vitro* .................................................................................................................. 46

2.4.3 5’-triphosphate-dependent PKR activation is biologically relevant and is observed *in vivo* ................................................................. 49

2.4.4 Effect of interferon-α production on 5’-triphosphate dependence *in vivo* ........................................................................................................... 52

2.5 Conclusions ........................................................................................................ 53

2.6 Acknowledgments ............................................................................................. 55

2.7 References ......................................................................................................... 55

**Appendix A**  Supporting Information: Chapter 2 .................................................. 59

A.1 Activation of PKR by 5’-diphosphate and 7mG-cap RNAs ............................ 66

A.2 Effect of siRNAs on PKR autophosphorylation .............................................. 67

A.3 Fluorescence polarization-detected binding assays ........................................ 70

A.4 Effect of RNA length and secondary structure on PKR autophosphorylation ................................................................................................. 71

A.5 Experimental determination of RNA secondary structures ......................... 74

A.6 Effect of 5’-triphosphate RNAs on eIF2α phosphorylation by PKR .......... 77

A.7 References ......................................................................................................... 81

**Chapter 3**  Characterization of the PKR 5’-triphosphate binding site ............. 84

3.1 Abstract ............................................................................................................. 84

3.2 Introduction ....................................................................................................... 85

3.3 Materials and Methods .................................................................................... 87

3.3.1 RNA preparation ............................................................................................ 87

3.3.2 Protein preparation ....................................................................................... 88

3.3.3 PKR activation assays .................................................................................. 88

3.3.4 PKR activation competition assays ............................................................... 89

3.3.5 Fluorescence competition assays ................................................................. 90

3.3.6 Isothermal titration calorimetry .................................................................... 91

3.3.7 Fluorescence detected stopped-flow kinetics .............................................. 91

3.4 Results and Discussion ................................................................................... 92

3.4.1 5’-ppp-dependent activation of PKR is not dependent upon nucleotide identity ......................................................................................... 92

3.4.2 Evidence for the existence of two NTP binding sites in PKR: the catalytic ATP site and the 5’-ppp binding site ................................................. 96

3.4.3 Base and ribose moieties are dispensable for binding to 5’-ppp binding site ................................................................................................. 100

3.4.4 Determination of binding affinity for ATP and GTP by competition fluorescence ..................................................................................... 102

3.4.5 Thermodynamic analysis of catalytic and 5’-ppp binding sites 105
Chapter 5  Effects of additional RNA structures on PKR function: (1) HCV IRES tertiary structure and (II) Non-Watson-Crick mismatches ... 176

5.A Main Abstract ................................................................. 176
5.1 Determining the role of an extended RNA tertiary structure on PKR activation by hepatitis C virus internal ribosome entry site (HCV IRES) RNA ................................................................. 176
5.1.1 Abstract ........................................................................ 176
5.1.2 Introduction .................................................................... 177
5.1.3 Materials and Methods .................................................. 181
5.1.3.1 RNA sequences ....................................................... 181
5.1.3.2 RNA preparation ..................................................... 182
5.1.3.3 Protein preparation .................................................. 183
5.1.3.4 PKR activation assays ............................................. 183
5.1.3.5 UV absorbance-detected thermal melting of RNA .... 184
5.1.4 Results and Discussion .................................................. 184
5.1.4.1 Mutations that disrupt RNA tertiary structure mitigate PKR activation by HCV IRES RNA ......................... 184
5.1.4.2 Mg$^{2+}$-dependence of PKR activation by WT IRES and tertiary mutants .............................................. 185
5.1.4.3 UV-dependent thermal denaturation of HCV IRES WT and mutants ..................................................... 189
5.1.4.4 Effect of double mutation on PKR activation by HCV IRES ............................................................... 191
5.1.5 Conclusions .................................................................... 193
5.2 Exploring the effect of tandem GA mismatches on PKR activation .... 195
5.2.1 Abstract ........................................................................ 195
5.2.2 Introduction .................................................................... 196
5.2.3 Materials and Methods .................................................. 201
5.2.3.1 RNA sequences ....................................................... 201
5.2.3.2 RNA preparation ..................................................... 202
5.2.3.3 Protein preparation .................................................. 203
5.2.3.4 PKR activation and inhibition assays ..................... 203
5.2.3.5 Native mobility gel-shift assays ............................. 204
5.2.4 Results and Discussion .................................................. 204
5.2.4.1 Effect of imino and sheared tandem GA mismatches in 33 bp dsRNA on PKR activation and inhibition . 204
5.2.4.2 Binding and inhibition of PKR by 16 bp RNA hairpins containing imino and sheared tandem GA mismatches ...................................................... 207
5.2.4.3 Effect of multiple tandem GA mismatches on PKR activation by 40 bp RNA ................................. 211
5.2.5 Conclusions ................................................................. 216
5.3 References........................................................................ 218
LIST OF FIGURES

Figure 1.1: Hierarchy of RNA folding. .................................................................3

Figure 1.2: RNA A-form helix and non-Watson-Crick base pair geometries. ....4

Figure 1.3: Function and mechanism of PKR in innate immunity. .......................6

Figure 1.4: Schematic of PKR domains and interactions. ....................................8

Figure 1.5: Structure of two dsRBMs from X. laevis Xlrbpa bound to dsRNA. ....10

Figure 1.6: Primary structural elements that regulate PKR. ...............................12

Figure 1.7: Activation of PKR by complex RNAs. .............................................15

Figure 1.8: Tertiary structural elements that regulate PKR. ...............................19

Figure 1.9: Interplay between the hierarchy of RNA folding and the activation of PKR. .................................................................................................................20

Figure 2.1: Activation of PKR by ss-dsRNA is 5'-triphosphate–dependent. .........32

Figure 2.2: Activation of PKR by ssRNA is 5'-triphosphate–dependent in vitro. ....48

Figure 2.3: Activation of PKR by ssRNA is 5'-triphosphate–dependent in vivo. ....51

Figure A.1: Efficiency of RNA dephosphorylation. ...........................................59

Figure A.2: Activation of PKR as a function of ss-dsRNA(9,11) concentration and treatment.................................................................60

Figure A.3: Activation of PKR by a mixture of 5'-triphosphate and CIP-treated RNA. ...............................................................................................................61

Figure A.4: 5'-triphosphate dependence for PKR activation by dsRNA and various ssRNAs. ...............................................................................................62

Figure A.5: Sequence and structure of ssRNAs based on lin-41. .........................64

Figure A.6: Activation of PKR by ssRNA-110 with various 5'-ends. ....................66
Figure A.7: Activation assays of PKR by siRNA duplexes and siRNA single strands. .................................................................68

Figure A.8: Activation and binding of PKR by ssRNAs of various lengths........69

Figure A.9: 5'-triphosphate dependence of PKR activation by ssRNAs having various stem-loop inserts.................................................................73

Figure A.10: Secondary structures of representative ssRNA-110 derivatives: ssRNA-76 and ssRNA-76-I38...............................................................75

Figure A.11: Activation of PKR by 5'-triphosphate RNAs leads to activation of eIF2α.................................................................78

Figure A.12: Activation of eIF2α correlates with activation of PKR................79

Figure A.13: Kinetics of PKR phosphorylation by various RNAs.......................80

Figure A.14: Kinetics of PKR and eIF2-α phosphorylation in response to dsRNA treatment .................................................................81

Figure 3.1: View of AMP-PNP bound in the PKR active site.........................86

Figure 3.2: Activation of PKR by ssRNA-47 with different starting nucleotides…94

Figure 3.3: The PKR catalytic site is specific for ATP.........................................98

Figure 3.4: Role of various NTP functional groups on 5’-ppp-dependent PKR activation determined by PKR activation competition assays. ..........101

Figure 3.5: Binding of ATP and GTP to K296R measured by fluorescence competition................................................................................104

Figure 3.6: ITC titration curves for NTPs binding to K296R. ..............................106

Figure 3.7: Kinetics of mant-ATP dissociation from K296R measured by fluorescence-detected stopped-flow..............................................108

Figure 3.8: Kinetics of mant-ATP dissociation from K296R upon competition with GTP or UTP measured by fluorescence-detected stopped-flow.110

Figure 4.1: Secondary structure of 5’-end of HCV RNA...................................122

Figure 4.2: Inhibition and activation of PKR by full-length HCV IRES RNA (1-388). .................................................................133
Figure 4.3: Activation of PKR by multiple domains of HCV IRES..............136

Figure 4.4: Activation of PKR and phosphorylation of eIF2α by multiple domains of HCV IRES. .................................................................139

Figure 4.5: Ribonuclease structure mapping of domain II..........................142

Figure 4.6: Footprinting of p20 onto domain II........................................145

Figure 4.7: Activation of PKR by domain II mutants.................................148

Figure 4.8: Native mobility gel-shift assays of NS5A binding to HCV domains..151

Figure 4.9: Inhibition of PKR activation by NS5A in the presence of domain II, domains III–IV, or 79 bp. .........................................................153

Figure 4.10: Structural comparisons of domain II with AGNN loop and 19 bp dsRNA. ................................................................................157

Figure B.1: Activation of PKR by domain II is not 5′-triphosphate-dependent...171

Figure B.2: Native mobility gel-shift assays of p20 binding to HCV domains. ...172

Figure B.3: Graphical representations domain II of footprinting data presented in Figure 4.6.A and B..........................................................173

Figure B.4: Activation of PKR by domain II mutants in L1, L2, and L3. .......174

Figure B.5: Activation of PKR and phosphorylation of eIF2α by 79 bp dsRNA in the presence of NS5A. ........................................................175

Figure 5.1.1: Crystal and NMR structures of HCV IRES tertiary domains in secondary structure context.....................................................180

Figure 5.1.2: Effect of HCV IRES tertiary mutants on PKR activation..........186

Figure 5.1.3: Mg2+-dependence of PKR activation by HCV IRES 1-388 wild-type and tertiary mutants. .........................................................188

Figure 5.1.4: UV absorbance thermal denaturation and derivative curves of HCV 1-388 WT, U228C, and G266C with varying Mg2+.....................190

Figure 5.1.5: Effect of tertiary double mutant (DM) on PKR activation........192
Figure 5.2.1: Hydrogen bonding within the two possible conformations of tandem GA mismatches: imino (left) and sheared (right).

Figure 5.2.2: Solution NMR structures of model RNA duplexes containing imino and sheared GA mismatches.

Figure 5.2.3: Activation and inhibition of PKR by 33 bp dsRNA containing one centrally positioned tandem GA mismatch.

Figure 5.2.4: Overlapping binding of dsRBM 1 and dsRBM 2 on a single dsRNA.

Figure 5.2.5: Native mobility gel-shift assays of p20 binding to RNA hairpins containing tandem GA mismatches.

Figure 5.2.6: Activation and inhibition of PKR by 16 bp dsRNA hairpins containing a tandem GA mismatch.

Figure 5.2.7: Inhibition of PKR activation by 16 bp dsRNA hairpins containing a tandem GA mismatch in the presence of a 40 bp dsRNA activator.

Figure 5.2.8: Activation and inhibition of PKR by 40 bp dsRNA containing three sets of tandem GA mismatches.
LIST OF TABLES

Table 3.1: Summary of functional assay results described in Figures 3.2-3.4 as applied to the 2-site model of NTP binding to PKR, where “Site 1” refers to the catalytic ATP binding site, and “Site 2” refers to the 5’-ppp binding site.................................................................111

Table 3.2: Summary of binding affinities determined by functional and biophysical assays described in Figures 3.3 and 3.5-3.6...............112

Table 4.1: Mediation of host and viral translation through NS5A-PKR interactions .................................................................................................................................163

Table 5.2.1: Summary of PKR inhibition results.................................................................217
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>79 bp</td>
<td>79 base pair RNA</td>
</tr>
<tr>
<td>7mG</td>
<td>7-methylguanosine</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>non-hydrolyzable ATP analog</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal phosphatase</td>
</tr>
<tr>
<td>dsRBD</td>
<td>dsRNA binding domain</td>
</tr>
<tr>
<td>dsRBM</td>
<td>dsRNA binding motif</td>
</tr>
<tr>
<td>DTT</td>
<td>dithioreitol</td>
</tr>
<tr>
<td>EBER</td>
<td>non-coding Epstein-Barr virus encoded RNA I</td>
</tr>
<tr>
<td>ECF</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemifluorescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetracetic acid</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic initiation factor 2a</td>
</tr>
<tr>
<td>G266C</td>
<td>HCV IRES mutant that disrupts tertiary structure at domain IIId</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GMP</td>
<td>guanosine monophosphate</td>
</tr>
<tr>
<td>HCV IRES</td>
<td>hepatitis C virus internal ribosome entry site</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIV TAR</td>
<td>human immunodeficiency virus transactivation-response region RNA</td>
</tr>
<tr>
<td>Huh-7, huh-7.5</td>
<td>human hepatoma cells</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IGA</td>
<td>imino tandem GA mismatch</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
</tr>
<tr>
<td>K296R</td>
<td>catalytically inactive mutant of PKR</td>
</tr>
<tr>
<td>λ-ppase</td>
<td>lambda phosphatase</td>
</tr>
<tr>
<td>mant-ATP</td>
<td>fluorescent ATP analog</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>NS5A</td>
<td>HCV non-structural protein 5A</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleotide triphosphate</td>
</tr>
<tr>
<td>p</td>
<td>monophosphate</td>
</tr>
<tr>
<td>p20</td>
<td>20 kDa double-stranded RNA binding domains of PKR</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>poly I:C</td>
<td>polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>pp</td>
<td>diphosphate</td>
</tr>
</tbody>
</table>
ppp, triphosphate (tripolyphosphate)

PSB, protein storage buffer

RIG-I, retinoic acid-inducible gene I

RMSD, root-mean squared deviation

Rnt1p-snR47h, small nucleolar substrate of Rnt1p endonuclease

SAXS, small-angle x-ray scattering

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

SGA, sheared tandem GA mismatch

siRNA, silencing RNA

ss-dsRNA, short double-stranded RNA with single-stranded tails

ssRNA, single-stranded RNA

TBE, 100 mM TRIS, 83 mM boric acid, 1 mM EDTA (pH 8.3)

TE, 10 mM TRIS (pH 7.5), 1 mM EDTA

TEK100, 10 mM TRIS (pH 7.5), 1 mM EDTA, 100 mM potassium chloride

TEN250, 10 mM TRIS (pH 7.5), 1 mM EDTA, 250 sodium chloride

TLR, Toll-like receptor

TNF-α, tumor necrosis factor α

TRIS, tris(hydroxymethyl)aminomethane

U228C, HCV IRES mutant that disrupts tertiary structure at junction IIIabc

UTR, untranslated region

VAI, virus associated non-coding RNA I from adenovirus

XXP, compound containing at least one phosphate group
ACKNOWLEDGMENTS

This work could not have been accomplished without the support of my family, friends, professors, and colleagues. At Franklin and Marshall College, I would like to thank my all of my professors in the Chemistry Department, and in particular my independent research advisor Dr. Ryan Mehl. Dr. Mehl introduced me to the world of research, and of biochemistry and molecular biology in particular, and continues to be a great mentor and friend.

At Penn State University I would like to than Dr. Andrew Ewing, Dr. Juliette Lecomte, Dr. Scott Showalter, Dr. Nicholas Winograd, and Dr. Craig Cameron for serving on my seminar, oral exam, and doctoral committees. In particular I would like to thank Dr. Cameron for being a wonderful collaborator and source of knowledge over the years.

I cannot imagine what graduate school would have been like without all of the amazing people I have worked with in the Bevilacqua lab. I was fortunate to have not one, but two incredible baymates: Dr. Andrea Szakal and Jenny Wilcox. Andrea, I continue to look up to you and am so grateful for all of your help and advice in my early years of graduate school. Jenny, I think you have become my partner in crime- thank you for your friendship and for keeping track of my schedule. To Josh Sokoloski and Melissa Mullen, thank you so much for being such incredible labmates and friends throughout all (or almost all) of my years of graduate school. I would also like to thank Dr. Durga Ghosh and Dr. Subba Rao Nallagatla for being terrific mentors and for never hesitating to answer my myriad questions and offer helpful suggestions. Thank you again to all
members of the Bevilaqua lab, new and old, for making it so much fun to come to lab every day.

I would like to thank Dr. Philip Bevilaqua for taking me into his lab my first summer here at Penn State. Phil, thank you so much for being a wonderful research advisor. I have learned so much from you over the years about how to be a better scientist and writer. Thank you for always encouraging me to tackle challenging problems and to not get discouraged when the data doesn’t look pretty (it’s because it’s real data!). I can only hope to strive for your passion and integrity as a scientist.

I would like to thank my family for all of your love, support, and encouragement. In particular, thank you to my siblings (and siblings-in-law!) for always being there for me, no matter what- I know I don’t always say it, but I love you guys and I appreciate more than I can articulate all you’ve done for me over the years- I wouldn’t have been able to accomplish any of this without you. Finally, thank you to my niece Elena for always being a source of joy.
Chapter 1

Introduction

[ Portions of this chapter have been previously published: “RNA Folding and the Regulation of Innate Immunity through the Protein Kinase PKR” by Subba Rao Nallagatla, Rebecca Toroney, and Philip C. Bevilacqua in Current Opinion in Structural Biology 2010 submitted; “PKR and the Ribosome Compete for mRNA” by Rebecca Toroney and Philip C. Bevilacqua in Nature Chemical Biology 2009 5: 873-874; and “A Brilliant Disguise for Self RNA: 5’-End and Internal Modifications of Primary Transcripts Suppress Elements of Innate Immunity” by Subba Rao Nallagatla, Rebecca Toroney, and Philip C. Bevilacqua in RNA Biology 2008 5: 140-144.]

1.1 Hierarchy of RNA Folding and Structure

In recent decades, RNA folding has been shown to play a role in an astounding number of cellular processes. In addition to its traditional role as an intermediary in translation, RNA can control gene expression by competing RNA base pairing and in response to metabolites through so-called riboswitches; small interfering RNAs (siRNA) can control transcription and translation through base pairing to target mRNA sequences; and RNA can even perform chemistry through self-cleavage and splicing reactions in ribozymes and the spliceosome, and through peptidyl transferase reactions in the ribosome.

The ability of RNA to participate in these diverse processes despite its limited
number of available functional groups can be attributed to the ability of RNA to adopt multiple, mutually exclusive stable folds. The folding of RNA has been described as hierarchical\textsuperscript{12,13} whereby folding proceeds sequentially from primary to secondary to tertiary, as depicted in Scheme 1.1:

\[ 1^o \rightleftharpoons 2^o \rightleftharpoons 3^o \]  

According to this folding pathway, primary sequence, which includes modifications to the bases internally or at the 5’-end, are transcribed first, with secondary structure forming as the RNA exits the polymerase, followed by formation of tertiary structure from the nascent secondary structure (Figure 1.1.A).\textsuperscript{13} Each level of the hierarchy is diverse: primary structure varies in sequence and length and may involve nucleotides with a variety of modifications to various functional groups located at the ends or within the strand (Figure 1.1.B). The A-form helix, which is discussed below, forms the basis of secondary structure and often involves multiple imperfections such as single and multiple nucleotide bulges, internal loops, and hairpins (Figure 1.1.C). Finally, tertiary structure involves higher order interaction of these helices (Figure 1.1.D) and can result in compact and globular or extended and flexible overall RNA structure.\textsuperscript{14} To add even further complexity, RNA elements at each level of hierarchy can interact with ions and other ligands, as well as proteins (Figure 1.1.E).\textsuperscript{15-17} This thesis focuses largely on the interaction of the RNA-binding protein PKR with RNA at each of these structural levels, and in particular on effects of secondary structure defects on RNA A-form geometry.

The RNA double helix adopts an A-form geometry, while DNA typically adopts B-form geometry. As a result, the major groove of RNA is deep and narrow, while the
Figure 1.1 Hierarchy of RNA folding. (A) Two-step folding pathway of a pseudoknot RNA, involving primary structure forming secondary structure, here a 5’-proximal hairpin, followed by tertiary structure, here interaction of the 3’-tail with the hairpin loop. (B) Primary structural elements of RNA, with certain 5’-end, 3’-end, and internal modifications provided. (C) Secondary structural elements of RNA, with perfect dsRNA, imperfections on one strand to give a bulge, on both strands to give an internal loop, or a stem-loop. (D) Tertiary structure elements of RNA, with coaxial stacking of helices, pseudoknot, and kissing hairpin loops depicted. (E) Binding of various species to RNA, with metal ion, ligand, and protein shown.

minor groove is wide and shallow (Figure 1.2.A). In addition to forming the two canonical base pairs (Figure 1.2.B), RNA adopts numerous non-Watson-Crick and mismatch interactions that can result in distortions to the shape of the A-form backbone. Indeed, more than 1800 different non-canonical base pair motifs have been annotated and
it is rare in eukaryotic RNAs to find an uninterrupted helix of more 7 bp.\textsuperscript{18,19} The most common non-Watson-Crick pairings are the G•U and A•C wobble (Figure 1.2.C), the latter of which requires protonation at N1. Wobble interactions occur at the Watson-Crick face, but the basepairing register is shifted. Other non-Watson-Crick interactions can occur at the Hoogsteen face, such as the GA sheared mismatch and AU Hoogsteen (Figure 1.2.D).

Figure 1.2 RNA A-form helix and non-Watson-Crick base pair geometries. (A) A-form RNA helix (PDB ID: 1QC0\textsuperscript{20}). Minor and major grooves are indicated. (B) Watson-Crick base pairs. Watson-Crick basepairing face is outlined in blue, and Hoogsteen face is outlined in red. (C) Non-Watson-Crick wobble base pairs of G•U and A•C, where A is protonated at the N1 position. (D) Non-Watson-Crick base pairs involving the Hoogsteen face of A in a sheared G•A and A in an A•U Hoogsteen.
Incorporation of non-Watson-Crick basepairs or mismatches into RNA helices often disrupts the A-form geometry through lateral widening of the deep major groove, although some non-Watson-Crick base pairs can be inserted with minimal helical distortion,\textsuperscript{21} thereby mimicking perfect helical base pairing. Non-Watson-Crick interactions and other helical imperfections often present alternative hydrogen bonding donor and acceptor sites for ligand and protein recognition, and impart flexibility to allow for formation of complex secondary and tertiary structure,\textsuperscript{22} thus contributing to the diversity of RNA structure and function.

1.2 Function and Structural Biology of PKR

The human double-stranded RNA-activated protein kinase, PKR, is a 551 amino acid interferon-induced serine/threonine kinase whose function is essential to the cell’s innate immune response.\textsuperscript{23,24} Innate immunity is the first line of host defense against viral infection and is mediated through virus recognition and signaling by the host cell, resulting in the disruption of virus replication.\textsuperscript{25} Many proteins are involved in this response in addition to PKR, including toll-like receptors (TLRs) and retinoic acid-inducible gene 1 (RIG-I).\textsuperscript{25} A key function of these proteins is distinguishing ‘self’ from ‘non-self’ through so-called pathogen-associated molecular patterns (PAMPs) in RNA that arise from the diversity of RNA sequence and structure.\textsuperscript{26} Through recognition and activation by non-self RNA, PKR and other proteins in the innate immune response induce downstream antiproliferative actions to combat viral infection.

The function of PKR in innate immunity is dependent upon autophosphorylation
induced upon non-sequence-specific interaction with RNA. In its latent, inactive state, dephosphorylated PKR undergoes autophosphorylation upon binding to activating RNAs (Figure 1.3) (discussed in the next section). Binding to these activating RNAs promotes ATP binding and phosphorylation at Thr446, followed by a cascade of phosphorylation events within the PKR kinase domain. Once in its phosphorylated state, PKR can then phosphorylate several different protein substrates, the most well-characterized of which is the eukaryotic initiation factor eIF2α. Phosphorylation of eIF2α at Ser 51 inhibits GTP-GDP exchange, ultimately leading to the shut-down of translation.
cellular translation and other antiviral effects (**Figure 1.3**). Viruses have evolved means of evading PKR activation, both through synthesis of non-activating RNA decoys, which sequester PKR in its inactive conformation (**Figure 1.3**), as well as production of pseudosubstrates. PKR in turn combats viral mimicry through rapid evolution of the PKR-substrate binding interface and through its ability to tolerate a wide variety of RNA structural imperfections.30-33

The structural biology of PKR is sparse, presumably due to the non-sequence-specific nature of its contact with RNA. PKR is unique among kinases in that, in addition to containing a C-terminal kinase domain, it contains a 20 kDa N-terminal RNA binding domain (also termed p20) that consists of two tandem dsRNA-binding motifs (dsRBM 1 and dsRBM 2) connected by a flexible 20 amino acid linker, which are collectively referred to as the dsRBD, or dsRNA binding domain (**Figure 1.4.A**).34,35 The NMR structure of the dsRBD has been solved in the absence of RNA and reveals an αββαβ fold for each dsRBM, which is common to many dsRNA-binding proteins (**Figure 1.4.A**, below schematic).17,36 The dsRBD is connected to the kinase domain by another linker region containing a mixture of acidic and basic residues, and the unstructured nature of this linker is believed to contribute to the ability of the kinase domain to dimerize, which is essential to PKR activation.37

The crystal structure of the kinase domain in dimer form bound to eIF2α has been solved in the absence of the dsRBD and the linker to the dsRBD, and consists of a smaller N-terminal lobe containing the dimer interface and a larger C-terminal domain that forms the contacts to eIF2α (**Figure 1.4.A**).28 The catalytic cleft is located at the interface of the two lobes and contains the activation loop typical of protein kinases, as
Figure 1.4 Schematic of PKR domains and interactions. (A) PKR domain organization and structures. N-terminal dsRNA binding domain of PKR (dsRBD) is composed of two dsRNA binding motifs (dsRBM 1 and dsRBM 2) connected by a flexible linker. The dsRBD is separated from the C-terminal kinase domain by a flexible region composed of acidic (-) and basic (+) residues. Position of K296R mutation, which renders PKR catalytically inactive, is indicated. NMR structure of dsRBD (also referred to as p20, PDB ID: 1QU636) and crystal structure of kinase domain dimer (eIF2α omitted for clarity, PDB ID: 2A1A28) with C and N lobes indicated, are pictured below the schematic. (B) Dimerization model for PKR activation by RNA. Binding of two PKR monomers to a single long dsRNA molecule promotes kinase dimerization and activation. (C) RNA concentration-dependent inhibition of PKR. At high concentrations of dsRNA, PKR monomers each bind to separate RNA molecules, thus inhibiting kinase dimerization. Figure is adapted from reference38.
well as the ATP binding site. A K296R mutation within the catalytic cleft inhibits phosphorylation at Thr446 and renders PKR catalytically inactive (Figure 1.4.A, schematic).

The molecular mechanism behind the dsRNA-based activation of PKR has been studied extensively, and several models have been proposed. These include the autoinhibition model, in which dsRNA binding to dsRBM1 releases PKR from an inactive conformation, and the dimerization model, in which dsRNA binding serves to promote kinase dimerization by bringing two PKR monomers into close proximity, although these models are not mutually exclusive (Figure 1.4.B, dimerization model). The latter model is supported by the characteristic bell-shaped dependence of PKR activation on RNA concentration, in which PKR is inhibited at high RNA concentrations, which can be attributed to titration of the active dimer into inactive monomers bound to separate RNA molecules (Figure 1.4.C). The dimerization model is also supported by analytical ultracentrifugation studies that have shown that the RNA length requirement of PKR activation (≥33 bp) correlates with the ability to bind two PKR monomers. In summary, the mechanism of PKR activation can be described by the following reaction scheme:

\[
2 \text{OH-PKR} + \text{dsRNA} + n\text{ATP} \rightleftharpoons 2 \text{PKR}\text{-dsRNA\text{-ATP}} \rightleftharpoons 2 \text{PKR(PO}_4\text{)}_n \\
\downarrow \text{eIF2α-PO}_4 \quad \rightarrow \quad \text{translation}
\]

in which two dephosphorylated PKR monomers dimerize on dsRNA of appropriate length and, in the presence of ATP, become phosphorylated at multiple sites, leading to phosphorylation of two eIF2α molecules (one bound to each PKR monomer), thus blocking cellular translation.
1.3 RNA Structure-based Regulation of PKR

1.3.1 dsRBM-RNA binding characteristics

Although no structure of PKR complexed with RNA has been solved, a crystal structure of the dsRBM from another dsRNA-binding protein, Xlrpba from *Xenopus laevis*, bound to A-form dsRNA provides insight into the molecular basis for PKR’s sequence-independent recognition of RNA (Figure 1.5). RNA bound structures of other dsRBM-containing proteins have also been solved, including Staufen, Rntp1 RNase II, and TRBP2 (transactivation response RNA-binding protein). In the Xlrpba structure, the two dsRBMs bind to separate faces of the RNA, with each spanning approximately 16 bp, or 1.5-helical turns. Dimerization of PKR requires a total of 4 dsRBMs binding to the same RNA. Shown in Figure 1.5.B is packing of two dsRBMs...
on ~20 bp of dsRNA, which implies that dimerization of PKR would lead to similar tight packing on RNA of ~33 bp, which biochemical studies have indicated is the minimum length of dsRNA required for PKR activation.\textsuperscript{48,49} Each dsRBM makes direct and water-mediated contacts with the RNA in three distinct regions: Regions 1 and 3 make minor groove contacts primarily to 2’-hydroxyls in the ribose sugar, while region 2 makes major groove contacts to phosphates in the RNA backbone (Figure 1.5.A).\textsuperscript{44} Extensive hydrogen bonding between the dsRBD and 2’-hydroxyls in the RNA backbone are consistent with the molecular specificity of PKR for RNA over DNA and DNA-RNA hybrids which do not contain sufficient 2’-hydroxyls in the appropriate pattern along the helix.\textsuperscript{36}

1.3.2 Primary structure-based regulation

As presented in section 1.1, the folding of RNA is largely hierarchical. Despite the designation of PKR as a dsRNA-binding protein, high information content exists at each level of the folding hierarchy regarding the RNA-based regulation of PKR. At the primary RNA sequence level, ssRNAs with small, ~5 bp stem-loops, and an imperfect 16 base-paired dsRNA with 10-15 nt single-stranded tails (so-called ‘ss-dsRNA’) activate PKR in a 5’-triphosphate dependent manner (Figure 1.6.A).\textsuperscript{50,51} This 5’-triphosphate functional group of ssRNA is key in PKR activation, as revealed by 5’-diphosphate, -monophosphate, -hydroxyl and 7mG cap-containing ssRNAs not activating PKR (see Chapter 2).\textsuperscript{51} Most endogenous cytoplasmic RNAs contain 5’-monophosphate or 7mG caps, generated through RNA processing,\textsuperscript{52,53} whereas bacterial and some viral RNAs contain 5’-triphosphates. The 5’-triphosphate functionality thus constitutes a PAMP for
PKR. Contrastingly, activation of PKR by dsRNA does not require a 5ʹ-triphosphate, indicating that PKR uses a different strategy for recognition of ssRNA and dsRNA.\textsuperscript{51}

**Figure 1.6** Primary structural elements that regulate PKR. (A) Structures of 5ʹ-triphosphate-dependent ss-dsRNA (top) and ssRNA (bottom two) PKR activators. (B) Schematic of modified nucleosides. Unmodified uridine (U) and adenosine (A) are depicted. Position of change in atom or functional group is shown.

Additional experiments have demonstrated that internal nucleoside modifications in 5ʹ-triphosphate ssRNA cause abrogation of PKR activation.\textsuperscript{54} Like at the 5ʹ-end, endogenous RNAs contain numerous *internal* modifications, whereas many exogenous RNAs do not. Specifically, minor groove modified nucleosides (s2U and 2ʹ-dU), major groove modified nucleosides (s4U, Ψ, m5U, I5U, and m6A), and Watson-Crick base-pairing-modified nucleosides (s2U, and s4U) abrogate activation of PKR by ssRNA (Figure 1.6.B). Remarkably, the minor groove-modifying nucleoside 2ʹ-FU, which
replaces the 2’-hydroxyl with a 2’-fluorine, retains the ability to activate PKR by this same RNA. The opposing effects of 2’-FU and 2’-dU support a role for hydrogen bonding between PKR and the ribose portion of ssRNA since fluorine can be a hydrogen bond acceptor.

Effects of nucleoside modification on PKR activation varied from ssRNA to dsRNAs, further supporting the hypothesis that ssRNA-based PKR activation is mechanistically distinct from dsRNA. By comparison, modifications to the minor groove in dsRNA affected PKR activation similarly as in the ssRNA, while modifications to the major groove, which abrogated activation in ssRNA, had either no effect or just slightly affected activation by dsRNA. Overall, these findings suggest that internal nucleoside modifications, like 5’-end specific modifications, modulate PKR activation in an RNA structure-specific context.26

1.3.3 Secondary structure-based regulation

Long stretches of dsRNA (≥33 bp) have long been proposed as the major activators of PKR. In the cell, these dsRNA activators typically arise during viral infection and are generated from viral genomes or replicative intermediates of RNA viruses, although transient dsRNA from transcription intermediates of DNA viruses can also activate.23 Early studies used perfect dsRNAs such as poly I:C and T7 transcribed dsRNAs of various lengths to characterize PKR activation.27 More recent studies have revealed activating RNAs that are non-perfectly double stranded, including various viral, cellular, and aptameric RNAs containing a wide variety of secondary structure imperfections. One such viral RNA is the human immunodeficiency virus
transactivation-response region (HIV TAR), a 23 bp hairpin RNA interrupted by three bulges that is also known to exist as a dimer (Figure 1.7.A).\textsuperscript{55} Although there has been some question about the role of HIV TAR RNA in regulation of PKR, recent evidence suggests that it is the dimeric form of TAR that is responsible for PKR activation.\textsuperscript{56,57} According to this model, two TAR hairpin monomers refold to form an extended duplex with two asymmetric bulges, which effectively doubles the number of base pairs relative to TAR monomer. By isolating TAR monomers and dimers via native gel electrophoresis, Heinicke et al. demonstrated that PKR activation by HIV TAR is strongly dependent upon TAR dimerization.\textsuperscript{57} Thus, oligomerization of shorter dsRNA can drive PKR activation by promoting binding of multiple PKR monomers on the same RNA.

Another strategy by which dsRNAs with imperfections can activate PKR is through structural mimicry of perfect A-form dsRNA, as demonstrated through PKR activation by domain II of hepatitis C virus internal ribosome entry site (HCV IRES) RNA. The IRES element of HCV has a highly complex secondary structure with four distinct structural domains containing multiple symmetric and asymmetric bulges, internal loops, and a pseudoknot. Despite these complicated structural elements, several domains of HCV IRES RNA have been reported as activators of PKR (see Chapter 4), including domains III-IV, which contains several multi-helix junctions and the pseudoknot, and domain II, a shorter hairpin region with internal loops and bulges.\textsuperscript{58,59} PKR activation by domain II in particular is surprising given these imperfections and its
Figure 1.7 Activation of PKR by complex RNAs. (A) HIV TAR RNA. Monomeric TAR contains only ~23 bp, which can bind only one PKR monomer, resulting in inhibition. Dimerization of two TAR monomers results in ~46 bp and promotes PKR dimerization and activation.\textsuperscript{57} (B) HCV IRES RNA, with domain II highlighted. Domain II contains fewer than 33 canonical and wobble base pairs, as well as several internal loops (left structures). Non-canonical pairings within these loops regions (right structure, depicted as dotted lines; green dotted boxes denote all nucleotides assigned to each loop) contribute to activation and provide the equivalent of ~33 bp of dsRNA.\textsuperscript{32}
limited number of canonical base pairs (≤ 33 bp). Footprinting and mutational analysis suggest that PKR is capable of binding and being potently activated by domain II RNA because the overall topology of its loop regions is primarily A-form.\textsuperscript{59} Non-Watson-Crick interactions between the bases in the loops of domain II maintain the A-form helical backbone geometry of these regions and contribute to the short stretches of canonical base pairs to sum to an activating total of ~33 bp (Figure 1.7.B). This work suggests that non-Watson-Crick base pairs within symmetric loops are well tolerated by PKR and that mimicry of A-form dsRNA may serve as a general mechanism for PKR activation by RNAs with multiple helical imperfections.

PKR regulation by RNA secondary structure is also typified by abrogation of PKR dimerization and activation through binding of inhibitory dsRNAs, such as those encoded by adenovirus (VA\textsubscript{i}) and Epstein-Barr virus (EBER\textsubscript{i}). Both RNAs bind PKR with similar affinity to activating RNAs but prevent PKR dimerization and subsequent autophosphorylation.\textsuperscript{60} VA\textsubscript{i} and EBER\textsubscript{i} have roughly similar structures (Figure 1.8.B) with three distinct domains: the apical stem-loop, the central domain, and the terminal stem. In the case of VA\textsubscript{i}, the apical stem-loop has been identified as the PKR dsRBD binding site and the three-way junction within the central domain is the determinant for PKR inhibition, which includes elements of tertiary structure that will be discussed in the next section.\textsuperscript{61} The terminal stem is completely dispensable for inhibition.\textsuperscript{62} The VA\textsubscript{i} apical stem-loop consists of ~18 canonical and non-canonical base pairs, which is sufficient for binding one PKR monomer but is not long enough to promote PKR dimerization. Interestingly, the apical stem-loop domain of VA\textsubscript{i} exists as a population of two conformations, one of which potently inhibits PKR the other of which displays
markedly decreased inhibition activity. The possible benefits of these functionally distinct structures for either the virus or host have yet to be determined.

Although the function of PKR is typically to serve as a sensor of viral or other non-self RNA, certain cellular RNAs have also been found to activate PKR. Previous work by Davis et. al. and Nussbaum et. al. identified the 3′-UTRs (untranslated region) of several highly structured cytoskeletal mRNAs as PKR activators. Similar to previously discussed viral RNAs, these cellular RNAs contain longer helical stretches interrupted by numerous bulges, internal loops, and branch points. Activation of PKR by cytoskeletal 3′-UTRs is predicted to play a role in the tumor-related activities of these sequences. Similarly, an element of the 3′-UTR of tumor necrosis factor α mRNA (TNF-α), which resembles the aforementioned ss-dsRNAs, has been shown to activate PKR.

Despite the strict dependence on RNA length, PKR activation by small interfering RNAs (siRNAs) containing only 19 to 21 bp has also been reported. A proposed model for PKR activation by these shorter RNAs suggested the possibility that PKR dimer assembled on one siRNA molecule can phosphorylate another PKR dimer bound to different siRNA molecule, although we have failed to replicate siRNA activation (Appendix A). This proposed model, however, does not appear to support the classic bell-shaped activation of PKR with RNA concentration, suggesting another mechanism may be operative. Conflicting reports in the literature suggest that experimental conditions, sequence, and helix termini of siRNA may play a crucial role in PKR activation, and further study is needed to sort out these discrepancies.
1.3.4 Tertiary structure-based regulation

Tertiary structure represents the final level of RNA hierarchy and serves as the basis for PKR activation by another cellular RNA, the 5'-UTR of interferon-gamma (IFN-γ) mRNA. As part of the interferon-mediated antiviral response, PKR participates in a negative feedback loop whereby IFN-γ regulates its own translation through competition between the ribosome and PKR for binding to IFN-γ mRNA$^{70-72}$. Depending upon relative levels of PKR in cell, the ribosome binds to IFN-γ mRNA to promote interferon synthesis (Figure 1.8.A). Upon clearing the ribosome, the 5'-UTR refolds to regenerate an RNA structure capable of activating PKR. Remarkably, IFN-γ mRNA contains four adjoining short helices that coaxially stack in a pseudoknot to cumulate to an activating total of ~33 bp. Thus, in addition to RNA oligomerization by HIV TAR and structural mimicry by HCV domain II, the amalgamation of secondary and tertiary features in IFN-γ mRNA demonstrates another means by which the hierarchical nature of RNA folding functions to generate RNA structures capable of activating PKR.

Finally, a less well-defined example of the role of RNA tertiary structure in PKR activation again lies in the inhibitory structure of VA$_1$ viral RNA. Recently, it was determined that Mg$^{2+}$, which is often required for stabilization of RNA tertiary structure, is required for correct folding of the VA$_1$ central domain.$^{73}$ Additionally, melting profiles and compensatory base pair modifications suggest a possible role of RNA tertiary structure in PKR inhibition by VA$_1$ RNA.$^{61}$ It has been suggested that while the terminal stem of VA$_1$ may function to stabilize this tertiary structure, in the absence of the terminal stem, PKR binding to VA$_1$ instead functions to stabilize the correct tertiary structure.$^{62}$
although the exact nature of this tertiary interaction has not been fully characterized.\textsuperscript{74}

\textbf{Figure 1.8} Tertiary structural elements that regulate PKR. (A) IFN-\(\gamma\) mRNA. The 124 nt 5-untranslated region is in blue, and the coding sequence is red. The ribosome and PKR compete for binding to IFN-\(\gamma\), with the ribosome disrupting the mRNA fold (depicted ribosome-bound fold is hypothetical). Pseudoknot formation as well as coaxial stacking of the surrounding helices leads to the equivalent of \(\sim 33\) bp of dsRNA and promotes PKR dimerization and activation.\textsuperscript{32} (B) Adenovirus VAI RNA. Secondary structure is depicted at left, with apical stem and central domain indicated (terminal stem omitted). Stem (S) and loop (L) elements are labeled. The tertiary fold of VAI is required for PKR inhibition through binding of one PKR monomer. A hypothetical tertiary structure of VAI with PKR bound is depicted, with stem and loop elements numbered and colored as per the secondary structure, and Mg\textsuperscript{2+} ion bound. Tertiary structure model is adapted from reference \textsuperscript{74}. 

---

19
1.4 Thesis Objectives

PKR was originally identified as a dsRNA binding protein. In the past decade, it has become abundantly clear that the RNA-based regulation of PKR is far more complex, as summarized in Figure 1.9. The goal of my thesis research has been to explore this interplay between RNA folding at all three structural levels and PKR activation in the context of viral and model RNAs.

Figure 1.9 Interplay between the hierarchy of RNA folding and the activation of PKR. The same overall two-step RNA folding pathway as present in Scheme 1.1 is shown. At each of the three states along the pathway, the potential for regulation of PKR by RNA exists. At the primary structural level, a 5'-triphosphate helps largely single-stranded RNA activate PKR, whereas a native 7mG cap and internal chemical modifications are incompatible with activation. At the secondary structural level, aggregates of RNA, depicted as ‘(RNA 2°)ₙ’, and certain RNA secondary structures activate PKR, while internal chemically modified RNAs and certain non-Watson-Crick motifs do not. Additionally, certain RNA tertiary structures, such as that of IFN-γ, appear to activate PKR, while others, such as that of the VA₁ RNA, do not.
PKR regulation by elements of RNA primary structure is explored in **Chapters 2** and **3**. **Chapter 2** describes the 5’-triphosphate-dependent activation of PKR by RNAs that are largely single-stranded and contain only one or two short ~5 bp stem loops. Activation by these RNAs displayed high molecular specificity, as the same RNAs containing 5’-diphosphate, -monophosphate, -hydroxyl or 7mG cap did not activate. The length dependence and importance and placement of the short stem loops was also defined. These results were also reproduced in human cell lines in collaboration with Dr. Craig E. Cameron’s laboratory.

This work is continued in **Chapter 3**, in which the location and specificity of the 5’-triphosphate binding site was investigated. The experiments performed in this chapter suggest the existence of two triphosphate binding sites within PKR, one of which is the catalytic site in the kinase domain that is specific for ATP, the other of which can bind any of the four NTPs. PKR competition activation experiments performed with both dsRNA and 5’-triphosphate-dependent ssRNA activators in the presence of NTPs revealed that only ATP can compete for PKR activation in the presence of dsRNA, which does not require a 5’-triphosphate for activation, while all four triphosphates compete for activation by ssRNA. This suggests that the 5’-triphosphate binds in a secondary, less specific site distinct from the ATP catalytic site. Additional experiments involving ITC, fluorescence competition, and fluorescence stopped-flow provide further evidence that the catalytic site is ATP-specific, which suggests that 5’-ppp’s binds in a second site that is likely weaker binding and more transient than the catalytic site, consistent with its weaker specificity.
**Chapters 4 and 5** investigate the role of RNA secondary structure imperfections and RNA tertiary structure in PKR activation. In **Chapter 4**, activation assays and footprinting experiments were employed to characterize PKR activation by the complex viral RNA, HCV IRES. Although a previous report indicated that HCV IRES RNA is an inhibitor of PKR, activation asays in **Chapter 4** performed over a broader range of RNA concentration confirm that HCV IRES, at the appropriate concentration, is also a potent activator of PKR. Individual secondary domains for the IRES were also tested for activation to determine which are primarily responsible for activation by the full IRES. Domain II was found to be an especially potent activator, and through footprinting and mutational analysis, it was determined that non-Watson-Crick interactions within the loop regions of Domain II contribute to PKR activation. Modeling indicated that A-form helical structural mimicry may be a strategy whereby RNA with helical defects can regulate PKR.

The effects of tertiary elements of HCV IRES on PKR activation are investigated in **Chapter 5**. It is observed that PKR activation is optimal when the tertiary structure is fully folded, as PKR activation is attenuated by HCV IRES containing mutations that interrupt elements of the tertiary structure. Finally, the role of tandem GA mismatches in PKR activation and inhibition are also studied in **Chapter 5**. Depending upon the flanking basepairs, tandem GA mismatches adopt one of two distinct geometries with opposing representation in biological RNAs: sheared or imino. Assays were performed in the context of model RNAs to determine if PKR is preferentially regulated by tandem GA mismatches containing the biologically rare imino geometry, which would suggest that this non-Watson-Crick motif serves as a molecular signal for PKR of non-self RNA.
A slight preference for PKR regulation by RNA containing imino GA mismatches is observed, although the fact that sheared GA mismatches also regulate suggests that rarity of phylogenetic occurrence of specific non-Watson-Crick motifs does not directly correlate with the ability to preferentially regulate PKR.

1.5 References


Chapter 2

5’-Triphosphate-dependent activation of PKR by RNAs with short stem-loops

[Published as a paper entitled “5’-Triphosphate-Dependent Activation of PKR by RNAs with Short Stem-Loops” by Subba Rao Nallagatla, Jungwook Hwang, Rebecca Toroney, Xiaofeng Zheng, Craig E. Cameron, and Philip C. Bevilacqua in Science 2007 318: 1455-1458.]

2.1 Abstract

Molecular patterns in pathogenic RNAs can be recognized by the innate immune system, and a component of this response is the interferon-induced enzyme RNA-activated protein kinase (PKR). The major activators of PKR have been proposed to be long double-stranded RNAs. We report that RNAs with very limited secondary structures activate PKR in a 5’-triphosphate–dependent fashion in vitro and in vivo. Activation of PKR by 5’-triphosphate RNA is independent of RIG-I and is enhanced by treatment with type 1 interferon (IFN-α). Surveillance of molecular features at the 5’ end of transcripts by PKR presents a means of allowing pathogenic RNA to be distinguished from self-RNA. The evidence presented here suggests that this form of RNA-based discrimination may be a critical step in mounting an early immune response.
2.2 Introduction

The innate immune response offers the host early protection from foreign organisms and viruses.\(^1\) As part of this response, the double-stranded RNA (dsRNA)–activated protein kinase (PKR) becomes activated through autophosphorylation in the presence of viral RNA.\(^2\) Subsequently, PKR phosphorylates eukaryotic initiation factor 2 (eIF2\(\alpha\)), which inhibits translation initiation, thus preventing pathogen replication.\(^2\)

PKR can be both activated and inhibited through its interaction with RNA, which is mediated by dsRNA-binding motifs (dsRBMs) (Figure 2.1.A) that also exist in other diverse proteins, including RNA-specific adenosine deaminases (ADARs), Dicer, and ribonuclease III.\(^3\) This interaction with dsRNA is sequence-independent,\(^4,5\) and although at least 16 base pairs (bp) of dsRNA are required for inhibition of PKR, 33 bp are needed for activation.\(^4,6\) We have previously shown that short dsRNAs with single-stranded tails (ss-dsRNAs) activate PKR, with the length of the tail providing a critical determinant.\(^6\) This motif has an imperfect stem of 16 bp and is flanked by single-stranded tails (Figure 2.1.B), and because it was prepared by transcription, it contains a 5′-triphosphate.\(^7\) This raises the question of what features of the tail might be important in activating PKR.
Figure 2.1. Activation of PKR by ss-dsRNA is 5'-triphosphate–dependent. (A) PKR has two dsRBMs (the dsRBD) and a kinase domain. (B) Experimental structures of ss-dsRNAs (6). (C) Activation assays using ss-dsRNAs [10% SDS–polyacrylamide gel electrophoresis (PAGE)]. RNAs were transcribed and untreated (5'-ppp) or CIP-treated (5'-OH). A no-RNA lane (--) is provided. Phosphorylation activities are normalized to 0.1 μM dsRNA-79 (no CIP).
2.3 Materials and Methods

2.3.1 Protein preparation

Full length PKR protein has an N-terminal (His)$_6$ tag that has been shown to not interfere with binding to and activation by RNA.$^{5,6}$ PKR and K296R were cloned into pET-28a (Novagen) and transformed into *E. coli* BL21(DE3) Rosetta cells (Novagen), and purified as previously described.$^5$ PKR was isolated in phosphorylated form, which was treated with λ-PPase (NEB)$^{6,8}$ before being subjected to phosphorylation assay. The concentration of proteins was determined spectrophotometrically.$^9$

2.3.2 RNA sequences

A series of dsRNAs, ss-dsRNAs, and ssRNAs were used in the study. Note that RNAs referred to as ssRNAs are done so because they do not contain a complementary strand. This is in accord with the literature on innate immunity,$^{10,11}$ and does not mean the RNAs do not form some secondary structure. These RNAs were prepared by transcription or chemical synthesis (Dharmacon) as described below. dsRNA-79 is a 79 bp dsRNA without base pairing imperfections derived from pUC-19$^6$ and was prepared by annealing equimolar concentrations of both ssRNA-79TS and ssRNA-79BS (see below). The siRNAs were prepared by T7 transcription with sequences from reference.$^{12}$ Sequences for the ss-dsRNA are from a previous *in vitro* selection experiment.$^6$ As an example of notation, ‘ss-dsRNA(9, 11)’ refers to RNA with 5’ and 3’-single stranded overhangs of 9 and 11 nts, respectively.
Most ssRNAs were derived from the 3’-UTR of lin-41, which is especially unstructured\(^\text{13,14}\) and were numbered according to length. A 110 nt portion of this 3’-UTR (ssRNA-110) was prepared, as was a 3’-end truncated 47mer (ssRNA-47) (**Figure A.5**). As an example of notation, ‘ssRNA-110’ refers to the 110 nt transcript from this 3’-UTR. Following are the sequences of RNAs used in this study. The 5’-end is indicated.

**dsRNA:** (The top strand (TS) is provided. The bottom strand (BS) is the Watson-Crick complement to the top strand and was annealed.)

- ssRNA-20TS: 5’GGGUUUCUGGUUUCGGUCU
- ssRNA-79TS: 5’GGGUUUUCCAGUCACGACGUUGUAAACGACGGCCAGUGAAUUCGC

**siRNA:** (top strand and bottom strand sequences were annealed)

- ssRNA-22TS: 5’GGCUGACCCUGAAGUCAUCA
- ssRNA-22BS: 5’GGUGAAGACUCUUAUCAGGGUCAGC
- ssRNA-24TS: 5’GGGGCUGACCCUGAAGUCAUCA
- ssRNA-24BS: 5’GGGGAUGACUUCAGGGUCAGC
- ssRNA-26TS: 5’GGGGAUGACUUCAGGGUCAGCA
- ssRNA-26BS: 5’GGGGAUGACUUCAGGGUCAGCU

**ss-dsRNA:** (ss tails underlined)

- ss-dsRNA(9,11): 5’GGGAGAGGUCACUGACUAAGUUGGUGAAAUCUUGAUUAUCAGUGAAGG
- ss-dsRNA(3,3): 5’GAGGUCACUGACUAAGUUGGUGAAAUCUUGAUUAUCAGUGAC

**ssRNA:** (ssRNA-20, -30, -40, -47 have a common 5’-end with ssRNA-110)
ssRNA-110:
5’GGCACCAACUCAAGUAUACCUUUUUAUACAAACCGUUCUACACUCAACGCGAUGUAAAUAUCGAUUCCCUUUUUAUACAAACAUUCUCUGCCUCUGGAAACAUUGGAAACCUCUUCU

ssRNA-21-AS (let-7):
5’UGAGGUAGUAGGUUGUAGAUAGU

**Stem-loop inserts into ssRNA-76** (a stem-loop with sequence, UGGACGAAAGUCCA was inserted into ssRNA-76 to give the following RNAs.)

ssRNA-76:
5’GGCACCAACACACAAAAACACAUCUACACUCAACACUACACUUUUUAUACAAACACUUCUCUUCUCACAUACACACUUCU-3’

ssRNA-76-15:
5’GGCACUGGACGAAAGUCCCAACACAAAACACAUCUACACUCAACACUACUCUCAACAAACACACUUCU-5’

ssRNA-76-113:
5’GGCACCAACACACAAUGGACGAAAGUCCCAACACAAUCUACACUCAACACUUCUUCUCUCUCACAUACACACUUCU-5’

ssRNA-76-121:
5’GGCACCAACACACAAUGGACGAAAGUCCCAACACAAUCUACACUCAACACUUCUUCUCUCUCACAUACACACUUCU-5’

ssRNA-76-131:
5’GGCACCAACACACACAAAACACAUCUACACUCAACACUGGACGAAAGUCCAAACAAUCUACACUUCUUCUCUCUCACAUACACACUUCU-5’

ssRNA-76-138:
5’GGCACCAACACACACAAAACACAUCUACACUCAACACUGGACGAAAGUCCAAACAAUCUACACUUCUUCUCUCUCACAUACACACUUCU-5’

ssRNA-76-146:
5’GGCACCAACACACACAAAACACAUCUACACUCAACACUACACUUUUUUAUACUGGACGAAAGUCCAAACACUUCUUCUCUCUCACAUACACACUUCU-5’

ssRNA-76-157:
5’GGCACCAACACACACAAAACACAUCUACACUCAACACUACACUUUUUUAUACAAACACUUCUUCUGGACGAAAGUCCAAACACUUCUACACACUUCU-5’
ssDNA:

ssDNA-47: DNA version of ssRNA-47, with Us changed to Ts

Cloning and vectors

DNA oligonucleotides were from IDT Inc., and were desalted prior to use. A plasmid was constructed by inserting a PCR product encoding ssRNA-110 into pUC19 vector. The PCR product was generated by overlap extension, which introduces an EcoRI cloning site upstream of a T7 promoter, a BsaI runoff site, and a BamHI downstream cloning site. Also, this PCR product has an additional BstUI enzyme recognition site that allowed run-off transcriptions of 47 nt. The following primers were used in overlap extension:

Primer1: 5’-CCGGGAATTCTAATACGACTCACTATAGGCACCAACTCAAGTGATACCTTTTATACAACCGTTCTACACTCAACGCGATGTAAATATCGCAAG

Primer2: 5’-GGCCGGATCCGGTCTCCAGAAGGTTCATGGTTCCAGAGGCAAAATGGTGTATAAAAAGGGATTGGCTATTTACATCGCGT

where the underlined sequences represent the overlap region

2.3.3 Preparation, purification, and CIP treatment of RNA

Chemically synthesized RNA oligonucleotides were purchased from Dharmacon. Transcribed RNAs were synthesized by T7 polymerase (Ambion) or by the following protocol: 1 µg of linearized template DNA or hemi-duplex DNA with T7 promoter sequence was incubated with 0.5 µg of T7 RNA polymerase in a buffer containing 40
mM Tris (pH 8), 20 mM Mg(OAc)$_2$, 40 mM DTT, 2 mM spermidine, and 7 mM of each NTP for 3 h at 37°C, followed by the removal of inorganic pyrophosphate by spinning the sample, and was quenched by the addition of 95%(v/v) formamide loading buffer. RNA was purified by denaturing polyacrylamide gel electrophoresis (PAGE). The transcript was identified by UV shadowing, excised from the gel, and eluted overnight. The RNA was concentrated by precipitation in ethanol, dissolved in TE buffer, and stored at -20°C. The concentration of RNA was determined spectrophotometrically.

To make the dsRNA-79 RNA, equimolar concentrations of purified ssRNA-79TS and ssRNA-79BS were annealed in TEN$_{100}$ [10 mM Tris (pH 7.5), 1 mM EDTA, and 100 mM NaCl] at a temperature of 95°C for 3 min. To make dsRNA-79 RNA that is devoid of 5’-triphosphate, each strand was treated separately with calf intestinal phosphatase (CIP) as described below and then annealed. Calf intestinal phosphatase removes the 5’-triphosphate and leaves a 5’-hydroxyl.$^{7,12}$ dsRNA-20 was prepared chemically (Dharmacon) and annealed as described for dsRNA-79. All siRNAs used were prepared by T7 transcription from hemi-duplexes. The siRNAs were made by annealing equal concentrations of TS and BS.

Calf intestinal alkaline phosphatase (CIP) treatment of RNA was performed as follows. A final concentration of RNA of 2 µM in 100 µL in 50 mM Tris (pH 7.9), 100 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT, was treated with 10 U of CIP (NEB) for 1 h at 37°C. The reaction was quenched by the addition of 8 µL of 0.5 M EDTA and heat inactivated at 75°C for 10 min. After CIP treatment, the solution was phenol-chloroform
extracted and the RNA was precipitated in ethanol and PAGE purified. $[{\gamma }^{32}P]$GTP-labeled RNA was prepared by T7 transcription (Ambion kit) in the presence of $[{\gamma }^{32}P]$GTP (10 mCi/ml; Perkin-Elmer), and the transcript was PAGE purified. To make the 7-methyl-G capped ssRNA-47 and -110, the following modifications were made to the transcription reactions. RNA was prepared with a 12:1 ratio of 7mGpppG:GTP by having 6.9 mM of 7-methyl-G cap reagent (Ambion), 0.58 mM GTP, 7.5 mM ATP, 7.5 mM CTP, 7.5 mM UTP in the transcription reaction. For making 8:1 and 4:1, the concentrations of cap reagent to GTP were adjusted in the transcription reaction accordingly. ssRNA-47 and -110 with a 5’-diphosphate were prepared in a similar manner, substituting GDP for 7mGpppG. ssRNA-110 with a 5’-monophosphate was prepared in a similar 12:1 manner, substituting GMP for 7mGpppG, while ssRNA-47 with a 5’-monophosphate was prepared by chemical synthesis (Dharmacon). All 5’-end modified RNAs were purified by PAGE.

2.3.4 PKR Activation Assays

Various RNAs were tested for their ability to activate or inhibit PKR autophosphorylation and eIF2α phosphorylation. PKR was first dephosphorylated by treatment with λ-PPase (NEB) for 1 h at 30 °C, followed by the addition of the freshly made phosphatase inhibitor, sodium orthovanadate. Next, 10 µCi $[{\gamma }^{32}P]$ATP (Perkin-Elmer), 0.6 µM dephosphorylated PKR and RNA were incubated in 20 mM HEPES (pH 7.5), 4 mM MgCl₂, 100 mM KCl, and 100 µM ATP (Ambion) for 10 min at 30 °C. The
time of 10 min was chosen because this is in the approach to the plateau region of phosphorylation versus time plots for dsRNA-79, ssRNA-47 and ssRNA(9,11), with all RNAs showing a lag as previously reported in the absence\textsuperscript{16} and presence of double-stranded RNAs (Figure A.13).\textsuperscript{17} In certain cases, 3 \(\mu\)M (10-fold excess) of purified eIF2\(\alpha\) was incubated for an additional 10 min. Reactions were quenched by adding SDS loading buffer. Samples were heated at 95\(^\circ\)C for 5 min and loaded on 10% SDS-PAGE gel (Pierce). Heparin, a known oligosaccharide activator, was used in certain experiments. Heparin was from Sigma and used without any further purification at a concentration of 1 \(\mu\)g/mL. After electrophoresis, the gels were exposed to a storage PhosphorImager screen and the intensities of the labeled bands were quantified on a PhosphorImager (Molecular Dynamics). In all experiments, 0.1 \(\mu\)M concentration of dsRNA-79 was included and the data were normalized to the counts of this dsRNA. A background value was averaged from different portions of the gel and subtracted from each band prior to normalization.

\textbf{2.3.5 Calculation of RNA-dependence for activation (RNA dependency factor)}

The dependence of a given PKR phosphorylation reaction on RNA was calculated as the intensity of a given band in the presence of added RNA divided by the intensity of the same band in the absence of added RNA. As noted above, all phosphorylation values reported in the figures were already corrected for background. PKR has the ability to weakly dimerize in the absence of RNA, which is the probable origin of the band in the
absence of added RNA.\textsuperscript{16} As a sample calculation, the RNA-dependency factor for
dsRNA-79 is calculated as $100/2.9 = 34.5$ (Figure 2.1.C).

\textbf{2.3.6 Calculation of 5’-triphosphate dependence for activation}

The dependence of a given PKR phosphorylation reaction on the 5’-triphosphate
was calculated as the intensity of a given band in the presence of a 5’-triphosphate-
bearing RNA divided by the intensity of the same band in the presence of a non-5’-
triphosphate- bearing RNA. The comparison is for the same RNA sequence at the same
concentration, and is calculated after subtracting the amount of phosphorylation in the
absence of added RNA from each value. Several sample calculations follow. The 5’-
triphosphate dependence for activation by 1 \( \mu \)M ss-dsRNA (as compared to 5’-OH
RNA) is calculated as $(100-2.1)/(2.9-2.1)=122$ (Figure A.2). This is reported in the text
as greater than 100 since only 1 significant figure is left in the denominator after
subtraction of 2.9 and 2.1. The 5’-triphosphate dependence for activation by 1.25 \( \mu \)M
ssRNA-47 (as compared to 5’-OH RNA) is calculated as $(63-1.7)/(2-1)=61$ (Figure 2.1).
Again, this is reported in the text as only 1 significant figure.

In certain cases, the measured 5’-triphosphate dependence represents a lower limit
because of potentially incomplete dephosphorylation by CIP, which could be caused by
the involvement of the 5’-most nucleotides in secondary structure, or incomplete priming
of transcription by a modified guanine nucleoside or nucleotide.
2.3.7 UV melting and mfold prediction

The thermodynamic stability of certain transcripts was determined by performing melting experiments in a melting buffer that mimics the PKR activation conditions (see Figure A.4.B and C). Melts were performed on a Gilford Response II spectrophotometer with a data point acquired every 0.5 °C and a heating rate of 0.6 °C/min at 260 nm from 5 to 95 °C. RNA was renatured in TE buffer at 95 °C for 1 min and then cooled on the bench for at least 10 min, followed by bringing the sample up in 1X melting buffer. In some experiments, the buffer was changed to 10 mM phosphate (pH 7.0), 100 mM KCl, 4 mM MgCl₂, and 0.1 mM EDTA. Cuvettes with pathlength of 1 mm were used, and the concentration of RNAs were in the 1-10 µM range. Melts were normalized by dividing absorbances collected as a function of temperature by the high-temperature absorbance.

2.3.8 Enzymatic structure mapping of RNA

5’-end labeled RNA was renatured by heating for 2 min at 85 °C followed by slow cooling to room temperature for at least 10 min. RNAs were digested with the appropriate nuclease under either native (15 min at 37 °C in PKR activation buffer- see ‘PKR activation assays’) or denaturing conditions (4 min at 90 °C in 100 mM Na₂CO₃/NaHCO₃, 2 mM EDTA for hydrolysis ladder, 30 min at 50 °C in 6 M urea, 17.8 mM Na-citrate at pH 3.5, 0.9 mM EDTA for T1 ladder). Nuclease concentrations were chosen to give limited RNA hydrolysis: 0.1 U/µL RNase T1, 0.1 ng/mL RNase A, 1.6 x
10^{-5}$ U/µL or $1.6 \times 10^{-6}$ U/µL RNase V1. Samples were fractionated on 12% denaturing 8.3 M urea polyacrylamide gels.

### 2.3.9 Fluorescence polarization analysis of binding

Synthetic ssRNA-47 was purchased from Dharmacon and 5’-labeled with fluorescein as follows. This method was adapted from reference.\textsuperscript{18} RNA was 5’-end activated with γ-S-ATP (Sigma) using T4 Kinase (NEB). After heat inactivation of the enzyme for 20 min at 65 °C, unincorporated γ-S-ATP was removed by gel filtration through G-25. The 5’-thiophosphorylated RNA was incubated with 5-iodoactamidofluorescein (Anaspec) in 100 mM Tris (pH 7.5) for 1 h at 37 °C. The 5’-fluorescein-labeled RNA was purified by 10% denaturing PAGE in reduced light. The percentage of labeled RNA was determined by UV-VIS measurement with a characteristic absorbance of fluorescein at 492 nm, as per manufacturer (Anaspec).

To determine the dissociation constant, ~0.07 nM of 5’-fluorescein-ssRNA-47 was incubated with varying concentrations of the catalytically inactive PKR mutant K296R in binding buffer [25 mM Hepes (pH 7.5), 8 mM Tris, 70 mM KCl, 10mM NaCl, 1.4 mM Mg(OAc)\textsubscript{2}, 5 mM DTT, 0.1 mM EDTA, 8.5% glycerol] at room temperature for 1 min, and the binding was monitored in terms of change in mp (millipolarization) on a Beacon 2000 fluorescence polarization system. We chose a catalytically inactive mutant of PKR so that it would not be phosphorylated either before or during the binding assay, as phosphorylation interferes with RNA binding.\textsuperscript{17,19} Equivalent mp readings were obtained
at 1 and 10 min, indicating the system was at equilibrium. For a blank measurement, a mixture containing all reagents except labeled RNA was used. All steps were performed in reduced light. The binding data were fitted to equation 1,

\[
mp = \varepsilon_{\text{max}} \frac{[P]^n}{[P]^n + [K_d]^n} + \varepsilon_{\text{min}}
\]

where \( mp \) is the millipolarization, \( \varepsilon_{\text{max}} \) and \( \varepsilon_{\text{min}} \) are the observed maximum and minimum millipolarization values, \( K_d \) is the dissociation constant, \( n \) is the Hill coefficient, and \([P]\) is the concentration of K296R.

In the competition experiments, varying concentrations of competitor RNAs were mixed with 0.07 nM of labeled ssRNA-47 prior to the addition of 160 nM K296R. The data were fitted to equation 2,

\[
mp = \varepsilon_{\text{max}} \frac{1}{2T_t} \left[ K_T + \frac{K_T}{K_C} C_t + P_t + T_t - \sqrt{\left( K_T + \frac{K_T}{K_C} C_t + P_t + T_t \right)^2 - 4T_t P_t} \right] + \varepsilon_{\text{min}}
\]

where \( P_t, T_t, \) and \( C_t \) are the total concentrations of protein, fluorescein labeled ssRNA-47, and unlabeled competitor RNA or DNA, respectively; \( K_t \) and \( K_c \) are the dissociation constants for ssRNA-47 and unlabeled competitor RNA or DNA complexes, respectively.

\[ 2.3.10 \text{ Cells} \]

Huh-7 and Huh-7.5 cells are human hepatoma cells. Huh-7, Huh-7.5 (provided by Dr. Charles Rice of Rockefeller University) and Vero (provided by Dr. Michael Teng of
Pennsylvania State University) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen), 100 units/ml penicillin/streptomycin (P/S, Invitrogen), and 0.1 mM non-essential amino acids (NEAA, Invitrogen).\textsuperscript{21}

2.3.11 Transfection

The transfection procedure was the same as previously reported.\textsuperscript{21} Briefly, cells were seeded at 6 X 10\textsuperscript{6} cells in 100-mm dish one day before transfection and incubated at 37 °C for 24 h. 1000 units/ml of interferon α (IFNα, Calbiochem) was added when required. 2 μg RNA in EC buffer with 4 μL Enhancer R was transfected into 1.6 X 10\textsuperscript{6} cells by TransMessenger kit (Qiagen). After 5 min incubation at room temperature, 8 μL TransMessenger was added to RNA and incubated for 10 min at room temperature (RNA complex). Cells were washed twice in media that did not contain any FBS, antibiotics or NEAA and resuspended in 900 μL media. After 10 min incubation, cells were added to RNA complex, which was rotated at 37 °C for 1 h. Cells were pelleted at 4000 rpm for 4 min and resuspended in media containing 10% FBS and 1% NEAA. Transfection was performed using liposomes, which can exhibit a slow, steady release of RNA.\textsuperscript{22} PKR-p accumulation was maximal at ~10 h post-transfection and persisted at least 20 h (Figure A.14). We therefore chose to incubate the cells for 8 h at 37 °C. Control (mock) experiments were performed using cells that were taken through the transfection protocol in the absence of RNA. Extracts were prepared from cells.
2.3.12 Western Blotting

The Western blotting procedure was similar to that previously reported. Briefly, transfected cells were lysed in SDS-PAGE sample buffer and boiled for 5 min. Samples were separated in 10% SDS-PAGE gel and proteins were transferred to nitrocellulose membrane (Amersham). The membrane was sequentially probed with eIF2α Ser-51 phosphorylation (Epitomics), PKR Thr-446 phosphorylation (Epitomics), and β-actin (Abcam) antibodies as indicated in figures. Secondary antibody was conjugated with horseradish peroxidase (Santa Cruz, for chemiluminescence) and was detected by using enhanced chemiluminescence (ECL, Amersham). In certain instances, enhanced chemifluorescence (ECF, Amersham) was used to provide semiquantitative analysis (data not shown). To reprobe with other antibodies, membranes were incubated in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50 °C for 30 min and washed. Background levels of eIF2α-p varied slightly between experiments.

2.4 Results and Discussion

2.4.1 Activation of PKR by ss-dsRNA is 5’-triphosphate-dependent, while PKR activation by dsRNA is not

In our initial experiments, we observed that a 79-bp perfectly dsRNA (dsRNA-79) led to potent activation of PKR, with an RNA-dependency factor of 35 (Figure 2.1.C). PKR was also activated by ss-dsRNAs and gave the expected 10-nucleotide (nt) tail-length dependence (Figure 2.1.C). Maximal activation by ss-dsRNA was as intense as
that by dsRNA-79, albeit requiring 10-fold more RNA. The ss-dsRNA(9,11) (having 5' and 3' tails of 9 and 11 nt, respectively) transcript (Figure 2.1.B) was next treated with calf intestinal phosphatase (CIP) to remove the 5'-triphosphate (Figure A.1), leading to abrogation of activation even at higher concentrations (Figure 2.1.C). Furthermore, chemically synthesized ss-dsRNA(9,11) having a 5' hydroxyl (Figure A.2) also failed to activate PKR. The presence of the 5'-triphosphate led to 100-fold higher PKR activation than occurred in its absence (Figure A.2). A mixture of CIP-treated and untreated transcripts showed full activation of PKR (Figure A.3), indicating that the reason why CIP-treated RNAs do not activate PKR is not because CIP treatment renders PKR incapable of activation.

To test whether the presence of 5'-triphosphate also affects the ability of long dsRNAs to activate PKR, top and bottom strands of dsRNA-79 were CIP-treated and annealed (Figure A.4). Unlike ss-dsRNA, CIP-treated as well as untreated dsRNA could activate PKR, with a standard bell-shaped dependence on RNA concentration (Figure A.4.D). Thus, long dsRNA does not require 5'-triphosphate, suggesting that the contribution of this motif to PKR activation is dependent on RNA structure.

2.4.2 Characterization of 1º and 2º structural elements in 5'-triphosphate-dependent activation of PKR by ssRNA in vitro

Given that ss-dsRNAs have functionally important non–base-paired elements, we next tested activation by the single strands of dsRNA-79, which also have secondary structure (Figure A.4.A and B). CIP-treated ssRNA-79TS (TS, top strand) and ssRNA-79BS (BS, bottom strand) transcripts were poor activators, whereas untreated transcripts
activated PKR at levels similar to those produced by dsRNA-79 (Figure A.4.E and F), with a measurable 5'-triphosphate dependence. The experiments thus far suggest that triphosphate makes the greatest contribution when the 5' end is unstructured. To examine this further, 47- and 110-nt transcripts with minimal secondary structure were prepared (Figure A.5), and despite having only a small number of short stem-loops, both transcripts activated PKR at levels close to those produced by dsRNA-79 (Figure A.4.G and H). CIP treatment greatly reduced activation, confirming dependence on 5'-triphosphate.

Many viral and bacterial RNAs possess 5'-triphosphates, whereas most cellular transcripts have a 7-methyl-guanosine (7mG) cap or a 5'-monophosphate. Thus, it is possible that PKR uses the 5' end of mRNA as part of a quality control mechanism. 5'-monophosphate or 5'-hydroxyl termini, prepared by chemical synthesis of ssRNA-47, were at least 50-fold less effective than a 5'-triphosphate terminus, yielding no detectable activation (Figure 2.2 and Appendix A.1). Transcripts primed with a 7mG cap or guanosine diphosphate (GDP) also failed to induce measurable PKR activation (Figure 2.2.B to 2.2.D). Parallel experiments on ssRNA-110 gave similar results (Figures A.4.G and A.6), indicating that longer transcripts also have a dependence on 5'-triphosphate for activation of PKR.
Figure 2.2 Activation of PKR by ssRNA is 5'-triphosphate–dependent in vitro. (A) Effects of 5'-OH and 5'-p on PKR activation. ssRNA-47 was synthesized with 5'-OH or 5'-p, and concentrations were 0.1, 0.5, 1, 2, 3, 5, and 10 µM (0.1 µM is omitted for 5'-p). (B) Effects of 5'-pp and 5'-ppp on PKR activation. ssRNA-47 was transcribed with guanosine triphosphate (GTP) only or with a GDP:GTP ratio of 12:1 and tested for PKR activation. RNA concentrations were 0.16, 0.31, 0.63, 1.25, 2.5, and 5 µM. (C) Effect of a 7mG cap on PKR activation. ssRNA-47 was transcribed with a 7mGpppG:GTP ratio of 4:1, 8:1, or 12:1. RNA concentrations were 0.16, 0.31, 0.63, 1.25, 2.5, and 5 µM. (D) PKR phosphorylation data from (C). The ratio of 7mGpppG:GTP was 0 (solid line, black circles); 4 (dashed line, white circles); 8 (solid line, black boxes); and 12 (dashed line, white boxes). Phosphorylation activities are normalized to 0.1 µM dsRNA-79 (no CIP).
Short 5’-triphosphate double-stranded small interfering RNAs, which induce an interferon response,\(^1\) as well as related short ssRNAs, failed to activate PKR (Figure A.7 and Appendix A.2). This suggested a minimal length of ssRNA required for activation, which we determined to be 47 nt (Figure A.8). In addition, a short (5-bp) stem-loop enhanced the magnitude of the response as well as the dependence on triphosphate (Figures A.9 and A.10). We also observed that the optimal positioning of the stem-loop in otherwise unstructured ssRNA was 21 to 46 nt from the 5’ end (Figures A.9 and A.10). One possible reason for this is that short stem-loops assist PKR binding, an idea that is supported by data on a dsRNA-binding domain (dsRBD) binding to 20-bp dsRNA that is consistent with a site size of 6 to 7 bp per dsRBD.\(^2\) As for other support, RNA/PKR binding assays revealed a correlation between RNA binding and kinase activation (Figure A.8.B and C).

### 2.4.3 5’-triphosphate-dependent PKR activation is biologically relevant and is observed in vivo

One of the biological substrates of PKR is eIF2\(\alpha\), the function of which in translation initiation is inhibited upon phosphorylation of Ser\(^51\).\(^2\) Upon activation by 5’-triphosphate ssRNA, PKR efficiently phosphorylated eIF2\(\alpha\) (Figures A.11 and A.12 and Appendix A.6), with each activated PKR molecule phosphorylating more than 100 eIF2\(\alpha\) molecules with a PKR:eIF2\(\alpha\) stoichiometry of 1:1. These results are consistent with a recent crystal structure, which showed each monomer in a PKR dimer interacting with an eIF2\(\alpha\) protein.\(^2\)  

To explore the biological relevance of our findings thus far, three cell lines were
selected to test components of the innate immune response: Huh-7, Huh-7.5, and Vero (Figure 2.3). All are responsive to interferon (IFN-α) but produce different levels of IFN-α/β in response to RNA virus (Figure 2.3.A). Transfection of dsRNA-79 into Huh-7 cells induced activation of PKR and increased levels of eIF2α phosphorylation (Figure 2.3.B, lane 2). Phosphorylated PKR was not observed in mock-transfected cells, although some eIF2α-p was detected (Figure 2.3.B, lane 1). For the ssRNA, a 110-nt oligomer was selected, which has the same 5′-end requirements as the 47-nt oligomer (Figures 2.2 and A.5), because longer RNAs possess superior transfection properties. Although 110-nt oligomer ppp-ssRNA (ppp, 5′-triphosphate) failed to activate PKR to a detectable level, a significant increase in eIF2α-p was nevertheless observed (Figure 2.3.B, lane 3) relative to mock-transfected cells. The response required the triphosphate, because the CIP-treated ssRNA transcript showed only background levels of eIF2α-p (Figure 2.3.B, lane 4). These results suggest that ssRNA requires a 5′-triphosphate for PKR-mediated phosphorylation of eIF2α intracellularly.

The helicase and innate immune sensor RIG-I has also recently been shown to be activated by ssRNA with a 5′-triphosphate.10,11 To determine whether activation of RIG-I contributes to PKR activation by 5′-triphosphate ssRNA, we used the Huh-7.5 subline of Huh-7, which lacks a functional RIG-I signaling pathway.28 Activated PKR was not detected in this cell line in response to dsRNA (Figure 2.3.C, lane 2) or ppp-ssRNA (Figure 2.3.C, lane 3), although a subtle yet reproducible increase in the amount of eIF2α-p was observed in response to dsRNA treatment (Figure 2.3.C, lane 2).
Figure 2.3 Activation of PKR by ssRNA is 5'-triphosphate–dependent in vivo. (A) Origin of cell line, capacity to produce IFN-α/β, and capacity to signal from the IFN-α/β receptor are indicated. A block of IFN-α/β production in Huh-7.5 cells is observed only if RIG-I signaling is required. The IFN-α/β gene cluster is deleted in Vero cells. (B to D) Cells were plated 24 hours before transfection in the absence (lanes 1 to 4) or presence (lanes 5 to 8) of 1000 units of IFN per milliliter. Cells were transfected with RNA (2 µg), prepared as for in vitro experiments. Proteins were denatured in SDS buffer and resolved by 10% SDS-PAGE. Phosphorylated PKR (PKR-p), phosphorylated eIF2α (eIF2α-p), and β actin (loading control) were identified by Western blotting. (E) Experiments were performed as in (B) using ssRNA-110. The 5' end was prepared by no treatment (ppp-lane). CIP treatment (HO lane), or priming transcription with a modified guanosine. (F) The 5' ends of dsRNA-79, ssRNA-79TS, and ssRNA-79BS were prepared as in (E). Samples in (E) and (F) were IFN-treated.
2.4.4 Effect of interferon-α production on 5'-triphosphate dependence in vivo

It was possible that a requirement for RIG-I for activation of PKR might be due to a need for IFN-α/β production. To test this, Vero cells, which are incapable of producing IFN-α/β, were tested, revealing activation of PKR by dsRNA (Figure 2.3.D, lane 2) but not ppp-ssRNA (Figure 2.3.D, lane 3). These data show that activation of PKR by dsRNA is independent of IFN-α/β production. Activation of PKR by ppp-ssRNA may use an alternative mechanism, requiring functional RIG-I signaling and/or IFN-α/β production.

All three cell lines we used are known to respond to IFN-α treatment (Figure 2.3.A). To further dissect the need for RIG-I signaling from IFN-α/β production, the experiments were repeated in cells treated with IFN-α. In all cell lines, dsRNA activated PKR on the basis of detection of both PKR-p and eIF2α-p (Figure 2.3.B to 2.3.D, lane 6). These experiments demonstrate that RIG-I signaling is not required for PKR activation by dsRNA. Likewise, ppp-ssRNA activated PKR in all cell lines treated with IFN-α, with both PKR-p and eIF2α-p being detected (Figure 2.3.B to 2.3.D, lane 7), indicating that RIG-I signaling is not required for PKR activation by ppp-ssRNA either. CIP-treated ssRNA did not produce significant levels of PKR-p, although this RNA led to some increase in eIF2α-p in Huh-7 cells (Figure 2.3.B to 2.3.D, lane 8), which is probably due to residual triphosphate. We conclude that ssRNA activates PKR in a 5'-triphosphate–dependent fashion in cells.

The data thus far are consistent with the existence of a "primed" form of cellular
PKR, which is induced by IFN-α treatment and required for effective activation of PKR by ppp-ssRNA. This conclusion is based on the observation that PKR activation by 5’-triphosphate ssRNA shows a strict dependency on IFN (Figure 2.3.B to 2.3.D, lanes 3 and 7) whereas PKR activation by dsRNA does not (Figure 2.3.B to 2.3.D, lanes 2 and 6). ppp-ssRNA is more potent than dsRNA in activating PKR in IFN-α–treated Huh-7 cells (Figure 2.3.B, lanes 6 and 7). Alternatively, it is possible that a higher PKR concentration, which is stimulated by IFN, is required for ppp-ssRNA–mediated activation of PKR.

### 2.5 Conclusions

For ssRNA-110, only a transcript with a 5’-triphosphate was capable of activating PKR intracellularly, and it also showed the greatest potency of activation of eIF2α (Figure 2.3.E). [The low levels of activation of eIF2α that were present for 5’-hydroxyl and the 7mG cap may be due to residual triphosphate (Appendix A.1).] In agreement with in vitro experiments, activation of PKR by ssRNA-79TS was 5’-triphosphate–dependent whereas activation by dsRNA-79 was not (Figure 2.3.F). Activation by ssRNA-79BS RNA was not 5’-triphosphate–dependent in cells; however, this transcript has a complex secondary structure (Figure A.4.A), which may facilitate the 5’-triphosphate–independent mode of activation. Indeed, a few RNAs with complex secondary structures are known to activate PKR, including RNA from hepatitis delta virus, the 3’ untranslated region from human alpha-tropomyosin, and various aptamers to PKR's dsRBD.30-32
The results presented here reveal that ssRNAs with very limited secondary structures have the ability to activate PKR in a 5'-triphosphate–dependent fashion. These activators differ from classical dsRNA activators. In particular, we have found that activation in a cellular context requires just 5 bp of RNA and is IFN-α–dependent but independent of RIG-I signaling.\textsuperscript{10,11} There is evidence that a number of ssRNA viruses use non-dsRNA to activate PKR in vivo.\textsuperscript{33} In particular, influenza virus has a 5'-triphosphorylated single-stranded viral RNA that activates PKR\textsuperscript{34} and does not produce detectable levels of dsRNA during replication.\textsuperscript{10} It is also notable that several virus families have evolved use of a protein primer that bypasses the presence of a triphosphate at the 5' end of RNA,\textsuperscript{23} which might represent a mechanism to evade PKR activation. 5'-triphosphate–dependent activation of PKR by ssRNA may be a major pathway for sensing and responding to viral infection \textit{in vivo}.

\textbf{Contributions:} SRN conducted \textit{in vitro} activation and binding experiments on ds, ss, si, and ss-dsRNAs containing various 5'-ends in text and Appendix A. JH conducted all \textit{in vivo} experiments in text and Appendix A. RT conducted all experiments involving length dependence of PKR activation and eIF2α phosphorylation, structure mapping, and design of model RNAs containing short stem-loop inserts at various positions, discussed in text and Appendix A. RT also contributed to all writing. XZ contributed primarily to design and early versions of experiments in text.
2.6 Acknowledgments

We thank B. Golden, D. Herschlag, and J. Reese for helpful comments and the following sources for funding: NIH grant GM58709 (P.C.B.) and the Martarano and Berg endowments of the Eberly College of Science (C.E.C).

2.7 References


Appendix A

Supporting Information: Chapter 2

[Published as Supporting Online Material for a paper entitled “5’-Triphosphate-Dependent Activation of PKR by RNAs with Short Stem-Loops” by Subba Rao Nallagatla, Jungwook Hwang, Rebecca Toroney, Xiaofeng Zheng, Craig E. Cameron, and Philip C. Bevilacqua in *Science* 2007 *318*: 1455-1458.]

![Image](image.jpg)

**Figure A.1** Efficiency of RNA dephosphorylation. In order to label the 5’-triphosphate, the following RNAs were transcribed in the presence of [γ-32P]GTP: ssRNA-47, ss-dsRNA(9,11), ssRNA-79TS, and ssRNA-79-BS. These RNAs were then treated with CIP under the standard conditions described in Chapter 2.3, Materials and Methods, which leads to dephosphorylation and release of radioactive inorganic phosphate, [32P]-p,
and loaded on the gel. The percent cleavage of starting material is provided under the gel. Dephosphorylation was highly efficient. The oligonucleotide RNA impurities were removed by PAGE purification prior to activation experiments.

Figure A.2 Activation of PKR as a function of ss-dsRNA(9,11) concentration and treatment (10% SDS-PAGE). In the first two sets of lanes, ss-dsRNA(9,11) was prepared as described in Chapter 2, Figure 2.1.C. In the third set of lanes, ss-dsRNA(9,11) was chemically synthesized to leave a 5’-hydroxyl. Concentrations of RNA were 0.1, 0.5, 1, 2, and 3 µM, except the 0.1 µM point was omitted in the third set of lanes. Phosphorylation activities for PKR were normalized to maximally active transcribed and untreated ss-dsRNA(9,11). The 5’-triphosphate dependence of activation (see Chapter 2.3.6) was calculated using the no RNA lane (-) and the two transcribed ss-dsRNA sets of lanes, because these were all run on the same gel.
Figure A.3 Activation of PKR by a mixture of 5’-triphosphate and CIP-treated RNA. RNA concentrations are noted in the figure. Equal concentrations of either HO-ssRNA-110 (obtained from CIP treatment of ppp-ssRNA-110) (Set 1) or 7mG-ssRNA-110 (after CIP treatment of 12:1 7mGpppG:GTP transcript) (Set 2) were mixed with an equal concentration of untreated ppp-ssRNA-110 and tested for PKR activation. Phosphorylation activities for PKR are normalized to 0.1 µM dsRNA-79 (no CIP).
Figure A.4 5'-triphosphate dependence for PKR activation by dsRNA and various ssRNAs. (A) Predicted secondary structures of ssRNA-79TS RNA and ssRNA-79BS RNA from mfold v3.2.1,2 (B) Normalized melting profiles of ssRNA-79TS (●) and ssRNA-79BS (○) and ssRNA-47 (◆). The melting profile for ssRNA-79BS RNA is offset by +0.02 units to aid visualization. For ssRNA-79TS and ssRNA-79BS, steep melting transitions with ~20% hyperchromicity and T_mS near 65 °C are observed, consistent with extensive and strong secondary structure. For ssRNA-47, a melting transition with only ~7% hyperchromicity, steep baselines, and a T_m near 45 °C are observed, consistent with limited secondary structure (Figure A.5.C, D). Melts were in 100 mM KCl, 2 mM Mg^{2+}, 20 mM Tris (pH 7.5), with RNA concentrations of ~8 µM. (C) Melting profiles of ssRNA-110 (1 µM) in the absence (●) and presence (○) of 3-
fold excess of the antisense RNA, ssRNA-21-AS. The melting profile in the absence of ssRNA-21-AS is offset by +0.02 units to aid visualization. For ssRNA-110, the melt is relatively featureless, consistent with even less percentage secondary structure than ssRNA-47, panel (B). For the ssRNA-110:ssRNA-21-AS complex, a steep melting transition with ~12% hyperchromicity and a $T_M$ near 60 °C are observed, consistent with ability to convert the unstructured ssRNA-110 to a highly structured RNA. Melts were in 100 mM KCl, 4 mM Mg$^{2+}$, 10 mM phosphate (pH 7.0), and 0.1 mM EDTA. (D) Activation of PKR by untreated (depicted as ‘ppp’) and CIP-treated (depicted as ‘OH’) dsRNA-79. Concentrations of RNA are provided in the figure. A no RNA lane is provided for reference. Phosphorylation activities for PKR are normalized to 0.1 µM dsRNA-79 (no CIP) for panels D-H. (E, F) Activation of PKR by untreated and CIP-treated ssRNA-79TS (E) and ssRNA-79BS (F). Concentrations of ssRNA-79TS and ssRNA-79BS were 1, 2 and 3µM. (G, H) Activation of PKR by untreated and CIP-treated ssRNA-110 (G) and ssRNA-47 (H). Concentrations of ssRNA-110, ssRNA-47, and dsRNA-79 were 1, 3, and 0.1 µM respectively.
Figure A.5 Sequence and structure of ssRNAs based on lin-41. (A) Sequence of ssRNA-110. The other lin-41-based ssRNAs studied have a common 5’-end and truncated 3’-ends. Every tenth nucleotide is in bold and numbered. (B) Predicted secondary structure of ssRNA-110. Free energy minimizations were carried out using mfold v3.2. (C, D) Experimental secondary structure of ssRNA-47. 12% denaturing gel is shown. Lanes are as follows. C is a control that was renatured at 90 °C and incubated in the absence of
nuclease at 37 °C for 15 min. HO- is a limited alkaline digest; T1 is a limited RNase T1 digest under denaturing or native conditions; A and V1 are limited digests with RNase A and V1, respectively. ‘Denaturing’ denotes treatment under denaturing conditions, while ‘Native’ denotes treatment under non-denaturing, PKR-activating conditions. Nucleotides located in structured regions are indicated to the right of the V1 lane. (D) Secondary structural model of ssRNA-47 derived from structure mapping experiments in panel (C) in conjunction with energy minimization from mfold v3.2. Positions of cleavage by single-stranded (RNase A, T1) and double-stranded (RNase V1) probes were assigned as previously described and are indicated as per the symbols provided in the figure.
A.1 Activation of PKR by 5’-diphosphate and 7mG-cap RNAs

After finding that ssRNA-47 with a 5’-triphosphate activates PKR, while ssRNA-47 with a 5’-OH or 5’-p does not (Figure 2.2.A and B), we tested RNAs with a 5’-pp or 7mG-cap. To prepare these RNAs, transcriptions were primed with modified guanosines, which led to a small amount of 5’-triphosphate RNA being present in the product. Transcriptions primed with GDP showed a significantly (~5-fold) reduced ability to activate PKR (Figure 2.2.B), with residual activation likely due to a small population of 5’-triphosphate transcripts. Transcriptions primed with 7mGpppG also displayed poor activation of PKR, with activation diminishing as the ratio of 7mGpppG to GTP in the transcription increases (Figure 2.2.C and D). These data suggest that neither a 5’-diphosphate nor a 7mG-cap on ssRNA can significantly activate PKR.

Figure A.6 Activation of PKR by ssRNA-110 with various 5’-ends. ssRNA-110 was transcribed in the presence of 12-fold excess of GMP, GDP, or 7mGpppG. RNA concentrations in all sets of lanes were 0.15, 0.31, 0.625, 1.25, 2.5, and 5 µM. Phosphorylation activities are normalized to 0.1 µM dsRNA-79 (no CIP).
A.2 Effect of siRNAs on PKR autophosphorylation

Recent reports indicate that transcripts of small interfering RNA (siRNA) of 19-21 base pairs, or ssRNAs of similar lengths, induce an interferon response in a 5’-triphosphate dependent fashion.\(^6\) On the basis of the results presented thus far, one possibility is that these RNAs operate by activating PKR. We therefore performed PKR activation assays on siRNA duplexes with 5’-triphosphates, as well as their component ssRNA transcripts, including some of those shown to induce interferon in the earlier study (Figure A.7).\(^6\) However, neither the siRNA duplexes nor their component ssRNA transcripts showed any evidence of PKR activation. These findings are in line with the length dependence of activation of PKR by ssRNA (Figure A.8) and dsRNA.\(^7\) The interferon response initiated by 5’-triphosphate siRNA appears to originate from a factor other than PKR, possibly RIG-I since it can be activated by 5’-triphosphate ssRNA as short as 19 nt.\(^8\)
Figure A.7 Activation assays of PKR by siRNA duplexes and siRNA single strands. (A) Sequences and base pairing registers of siRNA transcripts studied. siRNA-2 and siRNA-3 are designed from reference. Bold nucleotides do not have a Watson-Crick complement. (B) Activation assays for siRNA duplexes. All assays showed no evidence for activation.
(C) Activation assays for siRNA single strands. All assays showed no evidence for activation above background. For panels (B)-(C) concentrations of RNA were 0.1, 0.5, 1, 2, 3, 5, and 10 µM, and phosphorylation activities for PKR were normalized to transcribed dsRNA-79 (no CIP).

**Figure A.8** Activation and binding of PKR by ssRNAs of various lengths. (A) Dependence of phosphorylation on the length of ssRNA. ssRNAs were based on *lin-41*, transcribed with a common 5’-end with the activating 5’-triphosphate and various 3’-ends, and tested for activation of PKR. The lengths of the RNAs were 20, 30, 40, and 47 nt. Concentrations of RNAs were 0.16, 0.31, 0.62, 1.25, 2.5, and 5 µM. Phosphorylation
activities for PKR are normalized to 0.1 µM dsRNA-79 (no CIP). The minimal length of ssRNA for activating PKR is ~47 nt. (B) Fluorescence polarization binding assay for 5’-fluorescein ssRNA-47 (0.1 nM) binding to K296R (1.2 nM to 10 µM). Non-linear curve fitting gives a \( K_d \) of 0.14 ± 0.01 µM and a Hill coefficient of 1.7 ± 0.2. (C) Fluorescence polarization competition assay. The concentrations of K296R and 5’-fluorescein labeled ssRNA-47 were held constant at 160 nM and 0.05 nM, respectively, and challenged with increasing concentrations of unlabeled RNA and DNA, whose identities are given in the figure legend. The following RNA molecules, with their associated \( K_d \)’s, were able to compete with 5’-fluorescein-ssRNA-47 and return its polarization to its original value of ~100 mp shown in panel B: synthetic HO-ssRNA-47 (160 ± 130 nM), ppp-ssRNA-47 (94 ± 54), ppp-ssRNA-110 (34 ± 28), and dsRNA-79 (5.8 ± 7.5). Observation of approximately the same \( K_d \) for HO-ssRNA-47 by competition, as for 5’-fluorescein-ssRNA-47 by direct determination (in panel B) supports validity of the competition assay. This cluster of binding RNAs was given solid symbols and solid lines in their fits. Although the \( K_d \) values have substantial error associated with them, competition is certain as judged by the return of the polarization to its original value. The following RNA and DNA molecules were unable to compete effectively at concentrations up to 500 nM, as judged by their inability to return 5’-fluorescein ssRNA-47 to its polarization original value: ppp-ssRNA-30, HO-ssDNA-47, and 20 bp; this suggested their \( K_d \) values were greater than 0.5 µM. This cluster of RNAs could not be well fit and so symbols (open) were simply connected with lines (dashed) to aid visualization.

A.3 Fluorescence polarization-detected binding assays

One reason why short ssRNAs do not activate PKR is that they might not bind appreciably. To test this possibility, we carried out fluorescence polarization-detected binding assays (Figure A.8). Interaction between 5’-fluorescein-labeled ssRNA-47 and K296R, a catalytically inactive mutant of PKR, was strong with a \( K_d \) of 0.14 µM. Next, the ability of unlabeled RNAs to compete for binding was tested. We found that strong activators were strong competitors, while non-activators did not compete effectively. For
example, ppp-ssRNA-47 and -110 bound with $K_d$ of 94 and 34 nM, respectively, while non-activating ppp-ssRNA-30 had a $K_d > 500$ nM. In addition, activating dsRNA-79 competed strongly ($K_d$ of 6 nM), while non-activating 20 bp dsRNA did not ($K_d > 500$ nM). Interestingly, 20 bp dsRNA can bind to the dsRBD of PKR, suggesting that ssRNA activators may contact PKR at a site outside of the dsRBD. Also a 47 nt DNA, which does not activate PKR (Figure A.8.A), does not bind appreciably (Figure A.8.B); thus, recognition of ssRNA is sensitive to the nature of the ribose sugar, similar to dsRNA recognition. The 5’-triphosphate, which is critical for activation, had a modest 2-fold contribution to ssRNA-47 binding (Figure A.8.B and C). Increasing the Mg$^{2+}$ concentration to 10 mM did not appreciably affect the ability of ssRNA-47 to compete for binding either (data not shown). There is precedent in other systems for activating elements not leading to tighter binding, such as in the EcoRV endonuclease wherein favorable binding interactions with the cognate site are offset by concomitant activating distortions of the DNA.

**A.4 Effect of RNA length and secondary structure on PKR autophosphorylation**

We prepared 20, 30, and 40 nt truncated versions of the 47 nt ssRNA transcript, having the same 5’-end. Of these, only ssRNA-47 potently activated PKR (Figure A.8.A). This indicates that the minimal length of ssRNA needed to activate PKR is ~47 nt.

Although a 5’-triphosphate is important for ssRNA-mediated activation of PKR, it
is possible that other RNA features, such as secondary structure, are important for activation as well. We approached this problem in two ways. First, we prepared a series of RNAs with defined secondary structures. These have 5’-ends that are very similar in sequence to the activators ssRNA-47 and ssRNA-110. Initially, a 76 nt RNA (ssRNA-76) that does not engage in base pairing was engineered (Figure A.10.B). To prevent secondary structure, we designed ssRNA-76 with primarily A, C, and U residues; the only Gs present were at positions 1 and 2 to help drive T7 transcription, and consecutive C residues were disallowed in order to prevent any GC base pairing (with the exception of residues 5 and 6, which are too close to basepair with the G1 and G2) (Figure A.10.B). We chose to incorporate a mixture of A, C, and U residues rather than use a homopolymer because a mixture is more representative of cellular RNAs.

Absence of base pairing in ssRNA-76 was confirmed by structure prediction and experimentation. Structure prediction gave a minimum free energy of +3.4 kcal/mol, which indicates that the fully unstructured RNA populates to greater than 99%. Ribonuclease structure mapping experiments were also conducted on ssRNA-76, which revealed extensive single-stranded structure (Figure A.10.A). These data are consistent with the structural model shown in Figure A.10.B.

Next, we conducted PKR activation assays with ssRNA-76. These experiments revealed that ssRNA-76 is a modest activator of PKR with a weak 5’-triphosphate dependence (Figure A.9). These results suggest that certain secondary structures might be important for potent, 5’-triphosphate dependent activation of PKR.
Figure A.9 5'-triphosphate dependence of PKR activation by ssRNAs having various stem-loop inserts. Activation by the following 5'-triphosphorylated and CIP-treated RNAs were analyzed: ssRNA-76, ssRNA-76-I5, ssRNA-76-I13, ssRNA-76-I21, ssRNA-76-I31, ssRNA-76-I38, ssRNA-76-I46 and ssRNA-76-I57, where ‘I’ represents ‘stem-loop insert’ and number after ‘I’ represents the nucleotide position after which the stem-loop was inserted in ssRNA-76. The following 5 bp stem-loop was inserted into the RNA: 5’-UGGACGAAAGUCCA (stem is bold). Concentrations of RNAs used were 1.25 µM. Phosphorylation activities for PKR are normalized to 0.1 µM dsRNA-79 (no CIP).

We therefore engineered defined secondary structures into ssRNA-76, keeping the number of single-stranded residues constant. In this series of RNAs, a stem-loop with sequence, 5’-UGGACGAAAGUCCA (stem is bold) was inserted to give the following seven RNAs (where ‘Ix’, means stem-loop inserted at position x): I5, I13, I21, I31, I38, I46, and I57. This stem has 5 base pairs and the loop has a stable GAAA tetraloop with a CG closing base pair. The hairpin was predicted to be very stable, with a free energy of approximately -10 kcal/mol. We subjected a representative stem-loop RNA (I38) to structure mapping experiments, which revealed base pairing at the positions of interest (Figure A.10).
Next, we conducted PKR activation assays with the stem-loop insert RNAs. We found that if the stem-loop was located near the 5’- or 3’-end of ssRNA-76, activation was modest with a weak 5’-triphosphate dependence. However, if the stem-loop was located near the center of the RNA (21-46 nt from the 5’-end), potent and 5’-triphosphate dependent activation of PKR was found (Figure A.9).

A.5 Experimental determination of RNA secondary structures

In order to verify the structures of RNAs as predicted by mfold, we structure mapped three representative RNAs, ssRNA-76, ssRNA-76-I38, and ssRNA-47 using single- and double-strand-specific nucleases (Figures A.5 and A.10). For ssRNA-76, cleavages by V1 occurred near consecutive A’s, consistent with stacking of purines and the known ability of RNase V1 to cleave such regions.\textsuperscript{4,12} Additionally, many of the C’s in ssRNA-76 were cleaved by RNase A, suggesting that the RNA is largely unstructured. In the case of ssRNA-76-I38, which is predicted to have a stem-loop starting at U39, two double- stranded regions are present in the RNase V1 lane consistent with the 5’- and 3’-strands of this stem-loop. Cleavage by RNase A is not observed in this region.
Figure A.10 Secondary structures of representative ssRNA-110 derivatives: ssRNA-76 and ssRNA-76-I38. (A) Structure mapping of RNAs by RNase A and V1. Denaturing 12% polyacrylamide gel is shown, with all RNAs 5’-end labeled. Lanes are as follows: C1
is a control (no nuclease) that was not renatured or incubated at 37 °C; C2 is a control that was renatured at 90 °C and incubated at 37 °C for 15 minutes, consistent with nuclease treatment conditions; HO- is a limited alkaline digest; A and V1 are limited digests under native conditions with RNases A and V1, respectively. “Denaturing” denotes treatment under denaturing conditions, while “Native” denotes treatment under nondenaturing conditions. Nucleotides located in structured regions are indicated to the right of the V1 lane. (B, C) Secondary structural models derived from structure mapping experiments in panel A. Positions of cleavage by single-stranded (RNase A) and double-stranded-specific (V1) probes are indicated as per the symbols provided in the figure. (B) The most stable structure of this RNA has a predicted ΔG° of +3.4 kcal/mol,1,2 and so no base pairs are shown. Cleavages in the control lanes are consistent with known sites of reactivity in single-stranded RNA,13,14 while cleavages by RNase V1 are likely due to stacking of purines, which can lead to moderate RNase V1 activity.4,12 (C) Cleavages by RNase V1 are consistent with the stem-loop insert at position 38 and were assigned as previously described.4

The cleavage pattern of ssRNA-47 by RNase V1 is consistent with the predicted secondary structure in that the nucleotides cleaved by RNase V1 occur in the region of the two stem-loops. In particular, cleavage is observed in two sets of base pairs in the 5’-stem-loop, and on the 5’- and 3’-sides of the 3’-stem-loop. There are a few instances in both stem-loops wherein cleavage is observed by both RNase V1, a double-strand-specific nuclease, and RNases A and T1, single-strand specific nuclease, particularly at the very 3’-end of the RNA. The predicted and measured stabilities for both stem-loops provide melting near 37 °C (Figure A.4.B), which implies that folded and unfolded forms populate during structure mapping. The cleavage observed across all nuclease-treated and control lanes at certain sites (e.g. C28, C31, etc.) in both ssRNA-76 and ssRNA-76-I38 is likely due to spontaneous cleavage by 2’-OH attack, which has been observed to occur
preferentially in purine-pyrimidine dinucleotide stretches of single-stranded RNA.\textsuperscript{13,14}

Because these bands are not observed in ssRNA-47, which was treated at the same time and in the same manner as the ssRNA-76 and ssRNA-76-I38, these cleavage sites are inconsistent with nuclease contamination.

**A.6 Effect of 5’-triphosphate RNAs on eIF2α phosphorylation by PKR**

PKR activated by 5’-triphosphate ssRNA is a potent activator of eIF2α (Figures \textit{A.11 and A.12}). Optimal RNA concentrations for activating PKR led to similar extents of eIF2α phosphorylation, independent of RNA identity. A plot of phosphorylation activities for eIF2α and PKR has a slope of 7.7, indicating that there are ~8 eIF2α phosphorylations for each PKR phosphorylation (\textit{Figure A.12}). Since PKR is autophosphorylated at 15 or more sites,\textsuperscript{15-17} the turnover number for ssRNA-activated PKR is greater than 100. Thus, even a small amount of RNA with a 5’-triphosphate can have a potent effect on translation.

A log-log plot of the same data gives a slope of 1.0 (\textit{Figure A.12.C}), consistent with a recent structure of the kinase domain of PKR complexed with eIF2α in which PKR forms a homodimer, with each protomer interacting with one eIF2α.\textsuperscript{18} These data along with previous reports\textsuperscript{18,19} support a model in which ssRNA-activated or dsRNA-activated PKR sheds its RNA to exist as a dimer, with each protomer binding and phosphorylating one eIF2α.
Figure A.11 Activation of PKR by 5’-triphosphate RNAs leads to activation of eIF2α. PKR alone (left-hand set of lanes) or PKR plus eIF2α (right-hand set of lanes) were incubated with [γ-32P]ATP in the presence of various RNAs. Phosphorylation of PKR alone, or both PKR and eIF2α were assayed. The positions of phosphorylated PKR and eIF2α are noted. Concentrations of RNAs were near maximal values for PKR activation for that RNA and are provided in the figure. Phosphorylation activities for PKR in both sets of lanes were normalized relative to the dsRNA-79 lane in the absence of eIF2α, while phosphorylation activities for eIF2α were normalized relative to eIF2α band in the dsRNA-79 lane in the presence of eIF2α.
Figure A.12 Activation of eIF2α correlates with activation of PKR. (A) RNA concentration dependence of PKR and eIF2α phosphorylation. ssRNAs described in Figure S7, were tested for their ability to activate PKR and eIF2α as described in Figure S11. Concentrations of RNAs were 0.16, 0.63, 2.5 and 5 µM. Phosphorylation activities of PKR and eIF2α were normalized relative to the to eIF2α band in the dsRNA-79 lane in the presence of eIF2α; this normalization, which is different from that in Figure A.11, was done so as to visualize the molecularity with respect to PKR, and to highlight the ability of small amounts of phosphorylated PKR to lead to high levels of eIF2α phosphorylation. (B) Plot of normalized eIF2α phosphorylation versus normalized PKR phosphorylation, where the data were normalized as described in panel A. The plot has an R² value of 0.95 and a slope of 7.7, suggesting that there are on average ~8 eIF2α phosphorylation events for every PKR phosphorylation, or that ~100 eIF2α molecules are phosphorylated by each PKR molecule, as described above. (C) Plot of the logarithm of normalized eIF2α phosphorylation versus the logarithm of normalized PKR phosphorylation. The plot has an R² value of 0.92 and a slope of 0.998, suggesting that the molecularity of the reaction with respect to PKR is unity.
Figure A.13 Kinetics of PKR phosphorylation by various RNAs. 0.6µM PKR was incubated with 0.1 µM dsRNA-79 (no CIP) (▲), 3 µM ppp-ssRNA-47 (♦), 3 µM synthetic HO-ssRNA-47 (◊), 1µM ppp-ss-dsRNA (9,11) (●) and 1µM synthetic HO-ss-dsRNA (9,11) (○) in the presence of activation buffer. Aliquots were withdrawn as a function of time and quenched with SDS-loading buffer. Phosphorylated PKR samples were analyzed on 10% SDS-PAGE gels and quantified on a PhosphorImager (Molecular Dynamics). Activating RNAs displayed similar time courses, with an initial lag, and an approach to a plateau at 10 min.
Figure A.14  Kinetics of PKR and eIF2-α phosphorylation in response to dsRNA treatment. Huh-7 cells were incubated with 1000 units/ml IFN-α for 24 h followed by transfection with dsRNA-79. Transfected cells were incubated with RNA for 2, 5, 10, or 20 h. PKR-p, eIF2α-p, and β-actin (loading control) were detected by Western blotting. RNA activation was observed at all time points tested.

A.7 References


Chapter 3

Characterization of the PKR 5’-triphosphate binding site

3.1 Abstract

The interferon-inducible protein kinase PKR is an essential component of the innate immune system that functions through non-sequence-specific interaction with RNAs containing a wide variety of structural motifs, resulting in autophosphorylation that ultimately leads to inhibition of cellular translation. Although typical RNA activators of PKR are largely double-stranded in nature, there exists a unique class of activators that are mostly single-stranded with only minimal secondary structure. PKR activation by these RNAs, however, is strictly dependent upon the presence of a 5’-triphosphate group (5’-ppp) that is specific to non-cellular RNA; in this way, the 5’-triphosphate serves as a signal to the host to trigger the immune response. In this chapter, we report functional as well as thermodynamic and kinetic analyses of triphosphate binding to PKR. We find evidence for two triphosphate binding sites with differential NTP specificity and binding affinity, one of which functions as the catalytic ATP binding site, the other of which recognizes and binds RNA 5’-ppp. The characterization of the 5’-ppp binding site provides insight into the mechanism of PKR regulation by this class of atypical RNA activators and ability of PKR to distinguish between self and non-self RNA in general.
3.2 Introduction

Host recognition of molecular patterns in RNA serves as an integral component of the innate immune response in humans. A number of RNA sensors have been identified as part of this response, including Toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-I), and the protein kinase PKR. These sensors respond to specific pathogen associated molecular patterns (PAMPs) that distinguish RNA as non-self, such as long double-stranded stretches and internal nucleoside modifications. The 5’-triphosphate (5’-ppp) also has been identified as a PAMP for RIG-I and PKR, although the structural determinants of this activation are different for both proteins: RIG-I recognizes the 5’-ppp of short blunt-end dsRNA, while PKR recognizes the 5’-ppp of primarily ssRNA with short stem-loops. Because cellular RNA is typically capped by 7-methylguanosine at the 5’-end, the 5’-ppp serves as an indicator of pathogenic RNA.

PKR is typically activated by long stretches of dsRNA that result as intermediates of viral transcription and replication. This activation is mediated by two tandem N-terminal dsRNA binding motifs (dsRBMs). Autophosphorylation of PKR requires binding of two PKR monomers to dsRNA of at least 33 bp, which results in dimerization of the C-terminal kinase domains, thus promoting phosphorylation at Thr446. Given the requirement of 33 bp, PKR activation by 5’-ppp ssRNA with stem loops that can be as short as 5 bp was surprising (Chapter 2) and suggested a distinct mechanism of activation by these RNA is operable.

We previously identified and characterized the primary structural requirements for PKR activation by 5’-ppp-ssRNA in vitro and in vivo, and found that all three phosphates are required for activation, as ssRNA with 5’-end signatures of pp and p did not activate
Unlike RIG-I, dsRNA constructs have no requirement of this 5’-triphosphate for PKR activation. Additionally, we determined that PKR activation is optimal when the short stem loops are placed near the middle of the RNA. The mechanistic nature of these structural requirements for 5’-ppp-ssRNA activation remains unclear.

One most parsimonious hypothesis is that the 5’-triphosphate binds in the catalytic ATP binding site. This model thus assumes that a single triphosphate binding site within PKR serves two functions: 5’-triphosphate ssRNA recognition and catalysis. Inspection of the crystal structure of the PKR kinase domain with the ATP analog AMP-PNP bound in the catalytic cleft reveals steric accessibility for extension of an RNA chain from AMP-PNP’s 3’-hydroxyl (green, Figure 3.1). A alternative hypothesis assumes that there are two functionally distinct triphosphate binding sites within PKR. In the current study we seek to elucidate the mechanism of 5’-ppp-dependent PKR activation through functional and biophysical
characterization of the 5'-triphosphate binding site. The data presented herein support the latter model, wherein the catalytic binding site is highly specific to ATP, with tighter binding, while the 5’-ppp binding site is less specific, binding all NTPs with weaker binding.

3.3 Materials and Methods

3.3.1 RNA preparation

As described previously,\(^4\) dsRNA-79 was prepared by transcribing opposing strands of pUC19 and annealing. Wild-type (pppG-) ssRNA-47 (first nucleotide is G) was prepared by \textit{in vitro} by run-off transcription from a linearized pUC19 plasmid (\textit{Bst}U1 digested) containing a T7 promoter. For pppA-, pppC-, and pppU-ssRNA-47, the QuickChange site-directed mutagenesis kit was utilized to generate plasmid templates with A, C, or T mutations at the first nucleotide. Sequences were verified by dideoxy sequencing following maxipreps, and plasmids were digested as per pppG-ssRNA-47 template. For each RNA, 0.2 µg/µL of linearized plasmid was combined with 40 mM Tris (pH 8), 25 mM MgCl\(_2\), 2 mM DTT, 1 mM spermidine, 3 mM of each NTP, and 1 µM T7 polymerase containing a P266L mutation. Unlike wild-type T7 polymerase that generally requires G to prime transcription, this mutant polymerase has a more permissive promoter binding site and thus should allow incorporation of starting nucleotides other than G into transcripts, \(^11,12\)

After incubating 4 h at 37 °C, the reactions were quenched by addition of an equal volume of 95% (v/v) formamide loading buffer. RNA was then purified by fractionating on a polyacrylamide denature gel (7 M urea, 1X TBE). The transcript was identified by
UV shadowing, excised from the gel, and eluted overnight at 4°C in 1X TEN250. The RNA was then ethanol precipitated and resuspended in 1X TE buffer (pH 7.5) and stored at –20 ºC. RNA concentration was determined spectrophotometrically.

In order to verify that the correct starting nucleotide was incorporated into ppp-ssRNA-47, these RNAs were also transcribed in the presence of trace [γ-32P]ATP or [γ-32P]GTP. Because the radiolabel is on the gamma phosphate, a band should be observed only when the radioactive nucleotide is incorporated in the first position of the transcript.

### 3.3.2 Protein preparation

Full-length PKR and K296R (catalytically inactive mutant) containing N-terminal His6 tag were purified from *Escherichia coli* BL21(DE3) Rosetta cells (Novagen) as described previously. P266L T7 polymerase was prepared from *Escherichia coli* ENS0134T BL21 cells and purified on a Ni-NTA (Invitrogen) column, as described elsewhere.11,13 Protein concentrations were determined spectrophotometrically.

### 3.3.3 PKR activation assays

The ssRNA-47 transcripts containing various starting nucleotides were tested for their ability to activate PKR autophosphorylation. PKR was first dephosphorylated by treatment with λ-PPase (NEB) for 1 h at 30 ºC, followed by addition of the freshly made phosphatase inhibitor, sodium orthovanadate.14 Next, 15 µCi [γ-32P]ATP (Perkin-Elmer), 0.8 µM dephosphorylated PKR, and RNA at various concentrations were incubated in 20 mM HEPES (pH 7.5), 4 mM MgCl2, 50 mM KCl, and 100 µM ATP (Ambion) for 10 min at 30 ºC. In one experiment, designed to determine specificity for the catalytic ATP
binding site, 15 μCi [γ-32P]GTP and 100 μM GTP were added instead of [γ-32P]ATP and ATP. All reactions were quenched by addition of SDS loading buffer. Samples were heated at 95 °C for 5 min then loaded on 10% SDS-PAGE gels (Pierce). After electrophoresis, gels were exposed to a storage PhosphorImager screen and intensities of labeled bands were quantified on a PhosphorImager (Molecular Dynamics). A background value was averaged from different portions of the gel and subtracted from each band prior to normalization.

3.3.4 PKR activation competition assays

Various NTPs and other compounds were tested for their ability to compete for triphosphate binding sites during PKR activation by dsRNA and 5’-ppp-ssRNA. Activation assays were conducted as described above, with the following exceptions: RNA concentration was held constant in all lanes, and various concentrations of ATP, CTP, GTP, UTP, pyrophosphate (pp) , tripolyphosphate (ppp ), guanosine monophosphate (GMP) or guanosine diphosphate (GDP) (all from Sigma) were incubated along with other reaction components (see above). Gels were run and analyzed according to above. In the case of NTP competition, assays were performed in triplicate and data were normalized as described in Figure 3.2 caption. Error was calculated by averaging the percent PKR activation at each NTP concentration for each RNA and taking the standard deviation. Systematic error was estimated at 10% of the average value, and final error was determined from the square root of the sum of the squares of the two errors (standard deviation and systematic error). In the case of competition by ATP the data were fitted to Equation 3.1:
\[ \text{f}_{\text{PKR}} = A \left( \frac{1}{1 + \frac{[I]}{K_d}} \right) \]

where \( \text{f}_{\text{PKR}} \) is the fraction PKR bound, \( K_d \) is the dissociation constant for ATP, \( I \) is the concentration of ATP, and \( A \) is the observed minimum PKR activation.

### 3.3.5 Fluorescence competition assays

In order to determine affinities of unlabeled nucleotides for PKR, fluorescence competition assays were performed in which fluorescently labeled ATP (mant-ATP) and protein were prebound and held at a constant concentration while unlabeled competitor NTP (ATP or GTP) was titrated in increasing concentrations. All data were collected with a Fluoromax-3 fluorometer (Jobin Yvon Inc.) at room temperature in reduced light. Experiments were performed in protein storage buffer (PSB: 10 mM Tris (pH 7.6), 50 mM KCl, 2 mM MgCl\(_2\) and 7 mM ß-mercaptoethanol). Mant-ATP was diluted in 50 mM K\(_2\)HPO\(_4\) (pH 7.0). In order to avoid complications due to the undesired phosphorylation states of PKR, protein with a K296R mutation, which renders PKR catalytically inactive, was used at a constant concentration of 40 \( \mu \)M. Concentration of mant-ATP was kept constant at 10 \( \mu \)M (1:4, mant-ATP:K296R) or 80 \( \mu \)M (2:1, mant-ATP:K26R) and ATP or GTP was titrated in from ~12-2000 \( \mu \)M, with thorough mixing between each addition. To avoid diluting the sample during the course of the titration, a constant concentration of mant-ATP and K296R was added along with competitor NTP. Mant-ATP was directly excited at 360 nm and fluorescence emission was monitored at 443 nm. Data were fitted to Equation 3.2.\(^{15}\)
\[
I = I_{\text{max}} \frac{1}{2M_t} \left[ K_M + \frac{K_M}{K_C} C_t + P_t + M_t - \sqrt{\left( \frac{K_M}{K_C} C_t + P_t + M_t \right)^2 - 4M_t P_t} \right] + I_{\text{min}}
\]

where \( I \) is fluorescence intensity; \( P_t, M_t, \) and \( C_t \) are the total concentrations of protein, mant-ATP, and unlabeled competitor ATP or GTP, respectively; \( K_M \) and \( K_C \) are the dissociation constants for mant-ATP and unlabeled competitor ATP or GTP, respectively. The value for \( K_t \), 10.3 µM, is from reference 16.

### 3.3.6 Isothermal titration calorimetry

ITC was utilized to obtain thermodynamic parameters for NTPs binding to K296R. Data were collected using a MicroCal Auto-iTC200. The sample cell contained 40 µM K296R in PSB and the syringe contained 720 µM ATP, GTP, CTP, or UTP in PSB. Titrations were performed at 30 ºC and involved nineteen 2 µL injections into the 400 µL in the sample cell. The ATP titration curve was fitted to a model for two binding sites using MicroCal Origin software (Version 7.0).

### 3.3.7 Fluorescence detected stopped-flow kinetics

Kinetics of mant-ATP dissociation were measured using a Kintek fluorescence stopped-flow instrument (model SF-2001) fitted with a 450 nm bandpass filter, and mant-ATP was excited at 295 nm. This setup measures fluorescence resonance energy transfer (FRET) from Trp to mant-ATP. All measurements were performed in 20 mM HEPES (pH 7.5), 200 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, and 7 mM ß-mercaptoethanol. Dissociation kinetics were measured according to reference 16. Briefly, 2 µM
K296R was pre-equilibrated with 80 µM (or 100 µM) mant-ATP then mixed with 4 or 8 mM of a given NTP in a 1:1 (vol:vol) ratio, in order to promote dissociation of mant-ATP. Concentrations reported in text refer to final concentrations after mixing of the two syringes. For each trace, 500 time points were collected over 0.15 s and fitted to a one or two exponential decay function of the general form:

\[ y = A + Be^{-k_1t} + Ce^{-k_2t} \]

3.3

3.4 Results and Discussion

3.4.1 5’-ppp-dependent activation of PKR is not dependent upon nucleotide identity

Previous work in our lab has established that PKR activation by 5’-ppp-dependent ssRNAs is critically sensitive to the nature of the triphosphate moiety. In addition to abrogation of PKR activation by transcripts beginning with 5’-OH and 7-methylguanosine, transcripts beginning with 5’-p and 5’-pp were poor activators relative to the same RNA beginning with 5’-ppp (Chapter 2).\(^4\) The specificity for PKR activation of the base identity at position 1 of the transcript, however, has not been explored. RNAs transcribed \textit{in vitro} by T7 phage polymerase require G in the first position for transcription initiation,\(^12\) however bacterial and viral RNAs are initiated by G or A, and even occasionally by C.\(^17,19\) Thus, determination of base specificity for PKR activation has implications for both the structural nature of the 5’-ppp binding site and for the functional permissiveness of PKR activation by non-self 5’-ppp RNAs.

In an effort to overcome the restriction for a 5’-guanosine, we utilized a T7 polymerase containing a P266L mutation which has been shown to reduce abortive cycling during transcription due to decreased affinity of the promoter binding site.\(^11\) This
variant should thus be more tolerant to transcripts that start with nucleotides other than 5' -pppG, typically required by wild-type T7 polymerase (Olke Uhlenbeck, personal communication). We transcribed versions of ssRNA-47, which we have previously characterized as a strong 5' -ppp-dependent activator of PKR, with 5' -pppG (pppG-ssRNA-47), -pppA (pppA-ssRNA-47), -pppC (pppC-ssRNA-47), and ppp-U (pppU-ssRNA-47) starting nucleotides (Figure 3.2.A). To do so, we generated linearized plasmid templates corresponding to each of the four starting nucleotides via mutagenesis and transcribed the four RNAs in the presence of [γ-32P]ATP or [γ-32P]GTP in an effort to verify that the correct starting nucleotide was inserted (Figure 3.2.B). Because the label is located on the γ-phosphate, a band should be visible only when the labeled nucleotide is inserted at the first position. Thus, for [γ-32P]ATP, a band should be apparent only for RNA with A as the expected starting nucleotide, whereas for [γ-32P]GTP, a band should only appear for RNA with G as the expected starting nucleotide. Unfortunately, it was not possible to directly verify correct incorporation of C or U, because gamma-labeled versions of these nucleotides are not commercially available; nonetheless, we could verify lesser incorporation of [γ-32P]ATP and [γ-32P]GTP.

As shown in Figure 3.2.B, we observed preferential incorporation of [γ-32P]ATP and [γ-32P]GTP at position 1 where expected, and limited misincorporation of these nucleotides in transcripts starting with the other three nucleotides. It is noted, however, that transcripts starting with C and U transcribed poorly relative to transcripts starting with G or A (~3-fold lower yields). Thus, the observed limited misincorporation of [γ-32P]ATP
Figure 3.2 Activation of PKR by ssRNA-47 with different starting nucleotides. (A) Experimental structure of ssRNA-47. Starting nucleotide is depicted as ‘pppG’ (green), typical of T7 transcripts, but RNAs were also transcribed containing 5’-pppA, -pppC, and –pppU. (B) Verification of starting nucleotide identity. RNA was transcribed from DNA templates starting with 5’-pppG, -pppA, -pppC, and –pppT in the presence of [γ-32P]-ATP or [γ-32P]-GTP. Expected starting RNA nucleotide based on template (nt. 1: pppX) is indicated. The percent incorporation was calculated by normalizing the counts at the indicated mobility for each lane to the counts from the pppA lane (for [γ-32P]-ATP incorporation, LH 4 lanes) or the pppG lane (for [γ-32P]-GTP incorporation, RH 4 lanes). (C) Activation of PKR by ssRNA-47 with 5’-pppG, -pppA, -pppU, and -pppC. RNA concentrations were: 0.31, 0.63, 1.3, 2.5, 5.0, and 10 µM for pppG- and pppA-ssRNA-47; 0.15, 0.31, 0.63, 1.3, 2.5, 5.0, and 10 µM for pppU-ssRNA-47; and 0.15, 0.31, 0.63, 1.3, 2.5, and 5.0 µM for pppC-ssRNA-47. No-RNA and dsRNA-79 lanes are included. 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. Phosphorylation activities were normalized to the dsRNA-79 lane in the top gel and are provided under the gels. (D) Graphical representation of phosphorylation activites from panel (C) as a function of RNA concentration.
and \([\gamma^{\text{-}32}\text{P}]\text{GTP}\) cannot be fully attributed to high fidelity transcription of the correct starting nucleotide for \(\text{pppC-ssRNA-47}\) and \(\text{pppU-ssRNA-47}\).

We next tested these four RNAs for activation of PKR. We observed PKR activation of variable potency for \(\text{ssRNA-47}\) with all four \(5'\)-\(\text{pppNTPs}\) (Figure 3.2.C). Specifically, \(\text{pppG-ssRNA-47}\) activated maximally (88% the level of 79 bp activation) at 5 \(\mu\text{M}\), with a bell-shaped dependence on RNA concentration, as previously observed for this RNA (Chapter 2).

Maximal activation for RNAs with \(5'\)-\(\text{pppA}\) and \(5'\)-\(\text{pppU}\) was slightly less potent (1.1-1.2-fold lower) than for G, but their bell-shaped activation profile was shifted to lower RNA concentrations (1.25 \(\mu\text{M}\) RNA), implying higher affinity for these RNAs (Figure 3.2.D). Activation of PKR by both \(\text{pppA-ssRNA-47}\) and \(\text{pppG-ssRNA-47}\) is consistent with the prevalence of viral transcripts containing \(5'\)-G or \(5'\)-A, and, in particular, the more potent activation by \(5'\)-\(\text{pppA-ssRNA-47}\) is consistent with the highly conserved \(5'\)-\(\text{pppA}\) present in influenza A, B, and C viruses\(^{20,21}\) and the recently reported \(5'\)-\(\text{ppp}\)-dependence of PKR activation by influenza B virus ribonucleoprotein complex.\(^{22}\)

The \(\text{pppC-ssRNA-47}\) transcript was a less potent activator (up to 1.6-fold lower activation) than the other three RNAs and the bell-shaped profile was shifted to even higher RNA concentration than for \(\text{pppG-ssRNA-47}\), suggesting even weaker affinity for PKR. Although these data are consistent with the aforementioned prevalence of \(5'\)-end purines in human viral RNA, the potent activation by \(\text{pppU-ssRNA-47}\) is not. Given the precise overlay of the activation profiles for \(\text{pppA-ssRNA-47}\) and \(\text{pppU-ssRNA-47}\), it is possible that, as described for Figure 3.2.B, there may be misinsertion of \(\text{pppA}\) into the first position of the \(\text{pppU-ssRNA-47}\) transcript, thus resulting in a mixture of \(\text{pppA- and}\)
pppU-ssRNA-47 with pppA- primarily giving rise to the observed activation profile.

Overall, despite the variation in potency, PKR activation by ssRNA containing different base identities at the 5’-ppp suggests that the 5’-ppp binding site is permissive and that the contacts may be primarily to the triphosphate moiety and not the base.

3.4.2 Evidence for the existence of two NTP binding sites in PKR: the catalytic ATP site and the 5’-ppp binding site

One possible model for the 5’-ppp-dependent activation of PKR is that the 5’-triphosphate moiety of ssRNA binds in the ATP active site. This model, which is simplest in that it does not require a second binding site on the protein, is supported by steric accessibility at the active site for chain extension off the 3’-hydroxyl of ATP (Figure 3.1). Additionally, it has been shown that GTP and water can mimic ATP and bind in the active site of another protein kinase, CK2, suggesting that ssRNA with 5’-pppG, such as ssRNAs used in our previous study characterizing 5’-triophosphate-dependence of PKR activation (Chapter 2), could potentially bind in the catalytic site of ATP.

The activation results presented in the previous section and the reported 5’-triphosphate-dependent activation of PKR by a number of other ssRNAs with G as the starting nucleotide suggest that if the ATP active site is indeed the binding site for the 5’-triphosphate of ssRNA, then the active site may also be permissive to base identity for standard, dsRNA activation of PKR. Thus, we performed a PKR activation assay in which all ATP (unlabeled and [γ-32P]ATP) was replaced with GTP and [γ-32P]GTP.
shown in Figure 3.3.A, we observed complete abrogation of activation activity (Figure 3.3.A). This result indicates that the ATP binding site is base-specific for catalysis. Moreover, we also found that the \(\gamma\)-phosphate of 5'-ppp activators of PKR is not transferred to PKR (unpublished results). Thus, it appears that there are two binding sites for NTPs in PKR with differential specificity: one for ATP, and one for the 5'-triphosphate of ssRNA.

In order to further probe these two models, activation assays were performed in which PKR activation by a standard double-stranded activator, dsRNA-79, which does not require a 5’-ppp, and activation by pppG-ssRNA-47 (selected over pppA-ssRNA-47 because it was transcribed in higher yields) was competed by titration of each of the four NTPs in increasing concentrations (Figure 3.3.B). For dsRNA-79, only ATP was an effective competitor for PKR activation (up to 8.3-fold inhibition for the representative trial) which is reflective of unlabeled ATP competing out the labeled ATP from the active site. GTP, CTP, and UTP essentially did not compete for activation, with only up to 1.1-fold inhibition for each.

In the case of pppG-ssRNA-47, while ATP was a similarly effective competitor (again up to 8.3-fold), the other NTPs also competed for PKR inhibition. In particular, UTP and CTP inhibited up to 1.6- and 2.3-fold, respectively, while GTP inhibited up to 3.1-fold. Although these effects are still modest, a visual comparison between competition trends for dsRNA-79 (Figure 3.3.C) and pppG-ssRNA-47 (Figure 3.3.D) over the average of three trials illustrates the overall lack of competition for dsRNA-79 as
Figure 3.3 The PKR catalytic site is specific for ATP. (A) Ability of various NTPs to support PKR activation. Activation of PKR by dsRNA-79 using GTP or ATP as the phosphate source. RNA concentrations are indicated. In ‘γ-GTP’ lanes, activation assays were performed as per standard assay conditions (see Materials and Methods) with the following exceptions: 100 µM GTP was used instead of ATP, and 0.1 or 1.5 µCi/µL of [γ-32P]GTP was used instead of 1.5 µCi/µL [γ-32P]ATP. In ‘γ-ATP’ lanes, standard assay conditions were used. 10% SDS-PAGE gel is shown with position of phosphorylated PKR (p-PKR) indicated. No-RNA lanes are included. (B) Effect of NTP identity on PKR activation determined by activation competition assays. Representative assays of PKR activation by dsRNA-79 and pppG-ssRNA-47 in the presence of increasing concentrations of unradiolabeled ATP, GTP, CTP, and UTP. Concentration of dsRNA-79 was 0.1 µM in all indicated lanes, and concentration of pppG-ssRNA-47 was 2.5 µM. Concentrations of each NTP were 0.1, 0.5, 1, and 2 mM. No-RNA and no-competitor-NTP lanes are included. 10% SDS-PAGE gels are shown with position of phosphorylated PKR (p-PKR) indicated. Phosphorylation activities were normalized to the no-competitor-NTP lane in each gel and are noted under the gels. Note that in the middle gel, each RNA was normalized to its own no-competitor-NTP lane. Fold-effects relative to no-competitor-NTP are provided. Assay was performed in triplicate. (C) Graphical representation of phosphorylation activities for PKR activation by dsRNA-79 in the presence of competitor NTPs. Each data point is the average of three trials ± the standard error of the experiments. ATP trace was fitted to Equation 3.1, which gives a K_d of 157 ± 8. GTP, CTP, and UTP could not be well fit and so symbols were simply connected with lines (dashed) to aid in visualization. (D) Graphical representation of phosphorylation activities for PKR activation by ssRNA-47 in the presence of competitor NTPs as a function of NTP concentration. Each data point is the average of three trials ± the standard error. ATP trace was fitted to Equation 3.1, which a K_d of 280 ± 70. For GTP, CTP, and UTP, data could not be well fit and so symbols were simply connected with lines (dashed) to aid in visualization.

compared to pppG-ssRNA47 for GTP, CTP, and UTP. These data suggest that for ssRNA, the 5′-ppp is competed out of a site that is distinct from the catalytic binding and is also less specific.

In the case of ATP, competition for pppG-ssRNA-47 activation reflects binding at both the catalytic site, as was observed for dsRNA-79, as well as at the 5′-ppp binding site. The ATP traces for both RNAs were fit to the Equation 3.1 which is derived from
the following equilibrium:

$$E + I \rightleftharpoons EI$$ \hspace{1cm} (3.3)

where E is the concentration of PKR, I is the concentration of competitor NTP, and the fraction of PKR bound can be described according to:

$$f_{E_{\text{bound}}} = \frac{[EI]}{[E] + [EI]}$$ \hspace{1cm} (3.4)

The $K_d$ for dsRNA-79 (157 ± 8 µM) obtained from Equation 3.1 thus describes dissociation of ATP from the highly specific catalytic binding site, and for pppG-ssRNA-47, this value (280 ± 70 µM) is likely also dominated by dissociation from the catalytic site. It is noted that neither of the obtained $K_d$ values align well with reported values (~20 µM),\textsuperscript{16} but this is likely due to structural differences in PKR: ATP dissociation constants for PKR were previously measured in the absence of RNA, and thus PKR was likely in its monomeric state rather than its active, dimeric state as in the current assays. Additionally, determination of binding affinities through activation competition assays is a more indirect method than fluorescence competition assays used to obtain reported values.

### 3.4.3 Base and ribose moieties are dispensable for binding to 5'-ppp binding site

In order to further explore the relative specificity of the catalytic ATP and putative 5’-ppp binding sites, competition assays were performed with the following compounds as competitors: diphosphate (pp) and triphosphate (ppp), GMP, and GDP (Figure 3.4) Similar to competition by NTPs, GMP and GDP did not compete for dsRNA-79 activation. In the case of pppG-ssRNA-47, GMP did not compete for activation,
consistent with the previously established requirement of all three phosphates for 5'-ppp ssRNA activation. Competition was observed for ppp and, surprisingly, for GDP, with 2- and 3.4-fold inhibition, respectively. Competition for PKR activation by ppp alone provides further evidence for the limited specificity of the proposed 5'-ppp binding site, as it not only tolerates all four bases, but it apparently does not require a base at all for binding.

Figure 3.4 Role of various NTP functional groups on 5'-ppp-dependent PKR activation determined by PKR activation competition assays. (A) Representative assays of PKR activation by dsRNA-79 and pppG-ssRNA-47 in the presence of increasing concentrations of pyrophosphate (pp), tripolyphosphate (ppp), guanosine monophosphate (GMP) and guanosine diphosphate (GDP) (collectively termed ‘XXP’). Concentration of dsRNA-79 was 0.1 µM in all indicated lanes, and concentration of pppG-ssRNA-47 was 2.5 µM. Concentrations of pp, ppp, GMP, and GDP were each 0.5, 1, and 2 mM. No-RNA and no-competitor-XXP lanes are included. 10% SDS-PAGE gels are shown with position of phosphorylated PKR (p-PKR) indicated. Phosphorylation activities were normalized to the no-competitor-XXP lane in each gel and are noted under the gels. Fold-effects relative to no-competitor-XXP are provided. Assay was performed in duplicate. For both trials, some of the material in lanes containing pp did not migrate out of the wells. (C) Graphical representation of phosphorylation activities for PKR activation by dsRNA-79 in the presence of competitor XXPs as a function of XXP concentration. Each data point is the average of two trials.
Inhibition of pppG-ssRNA-47 activation by GDP is unexpected due to previously published results in which replacement of 5’-ppp with 5’-pp in a 5’-ppp-dependent ssRNA abrogated activation. It is possible that while two phosphates are sufficient to interfere with productive 5’-ppp binding, as per Figure 3.4.A and B, RNA with only two 5’-end phosphates cannot bind productively for activation, much in the way that classical dsRNA activators of PKR that are too short to promote activation can sometimes serve as potent inhibitors.

For both RNAs, pp was a potent competitor for PKR activation; however, a smear was observed in both gels for both trials in the pp that extended up into the wells. Thus, the decrease in band intensity is actually an artifact due to a portion of the material being stuck in the wells, although the reason for this retarded migration remains unclear.

### 3.4.4 Determination of binding affinity for ATP and GTP by competition fluorescence

To further investigate the idea that PKR contains two binding sites for NTPs, competitive fluorescence titrations were performed. These assays are based on experiments performed by Lemaire et al. in which a fluorescent ATP analog, mant-ATP, was employed to characterize dissociation of ATP from various forms of PKR (i.e. phosphorylated, unphosphorylated, K296R). We utilized the same fluorescent analog, which is known to increase in quantum yield upon protein binding, to measure the affinity of binding of ATP and GTP to PKR (Figure 3.5.A). To avoid complications from the phosphoryl transfer reaction, the catalytically inactive mutant, K296R was used for these studies.

Fluorescence competition assays were performed by titrating ATP or GTP against
K296R pre-incubated with mant-ATP at fixed concentrations. Mant-ATP was directly excited at 360 nm and fluorescence emission was monitored at 443 nm, and data were fit to **Equation 3.2**. First, the assay was conducted with K296R in 4-fold excess over mant-ATP. As expected based on PKR activation competition assays, ATP is an effective competitor for mant-ATP binding, with a $K_d$ of 47 ± 4 (Figure 3.5.A). This observed $K_d$ is approximately two-fold weaker than reported values (~20 µM) determined by competitive anisotropy titration by Lemaire *et al.*, but the difference in these $K_d$ values could be attributed to differences in Mg$^{2+}$ concentration between our assays (2 mM Mg$^{2+}$) and those (5 mM Mg$^{2+}$). The lower Mg$^{2+}$ concentrations in our assays would result in less coordination to the ATP phosphate groups, and two of the crucial binding interactions between PKR and ATP in the catalytic site are mediated by Mg$^{2+}$ ions. Thus, the lower Mg$^{2+}$ concentrations in our assays could result in the weaker observed binding affinity. Consistent with PKR activation competition assays, GTP was a weaker competitor for mant-ATP binding, with a $K_d$ of 510 ± 350 µM (Figure 3.5.A).

As mentioned, in the previous assay, K296R is in 4-fold excess over mant-ATP. If PKR contains two NTP binding sites, one of which has 10-fold tighter binding ($K_d$ for GTP/$K_d$ for ATP from previous assay) under mant-ATP-limiting conditions it is possible that mant-ATP fills the tighter binding site with the weaker binding site remaining essentially empty. We thus repeated the competition experiment with mant-ATP in excess 2-fold over K296R (Figure 3.5.B). In this case, two phases are indeed observed for competition with ATP. The first phase gives a $K_d$ of 34 ± 4, which is in line with the values measured with limiting mant-ATP. While the second phase did not fit well to **Equation 3.2**, it appears to represent dissociation at a weaker binding site. Competition
by GTP was again very weak relative to ATP (Figure 3.5.B, inset). These data support
the presence of two NTP binding sites within PKR: (1) a tighter binding site that only
binds ATP and functions in catalysis, and (2) a weaker site that can be competed by ATP
and GTP which is involved in PKR activation by 5’-pppRNA.

Figure 3.5 Binding of ATP and GTP to K296R measured by fluorescence competition.
(A) ATP (■) or GTP (●) was titrated against 10 μM mant-ATP pre-bound to 40 μM
K296R (1:4 mant-ATP:K296R). Fluorescence emission was monitored at 443 nm at an
excitation of 360 nm. Each data point represents the average of two trials. Data were
fitted to Eq. 3.1, with a Kₐ for ATP of 50 ± 4 and a Kₐ for GTP of 510 ± 350 μM. The Kₐ
d of binding of mant-ATP to K296R used in the fitting equation was 10.3 μM.16 (B) ATP
(■) or GTP (●, inset) was titrated against 80 μM mant-ATP prebound to 40 μM K296R
(2:1 mant-ATP:K296R). Each data point represents one trial. Data revealed tight and
weak binding arms. Data for tighter binding arm were fitted to Eq. 3.2, with a Kₐ of 34 ±
4 μM.
3.4.5 Thermodynamic analysis of catalytic and 5'-ppp binding sites

To further investigate the idea that PKR contains two binding sites for NTPs, isothermal titration calorimetry (ITC) was employed. Affinities and stoichiometries of NTP binding to K296R were determined (Figure 3.6). These experiments again utilized K296R in the absence of RNA, with injections of NTP into a concentrated protein sample (40 µM). The titration curve for GTP revealed a very weak interaction that was barely noticeable in comparison to titration against buffer, and likewise UTP and CTP titration curves were essentially equivalent to the buffer titration (Figure 3.6.A). In contrast, the ATP titration curve was best fit to a two-site binding model. The first site revealed an exothermic interaction with a $K_d$ of ~4 µM and $n = \sim 1$, supporting a 1:1 stoichiometry of K296R to ATP, as expected based on the crystal structure of PKR (Figure 3.6.B). This binding affinity is in agreement with the $K_d$ determined herein by fluorescence competition.

The second site was lower in affinity ($K_d \sim 20$ µM), with a binding stoichiometry of $n = 0.1$ (Figure 3.6.B). Given the high affinity at both sites as determined by ITC, it seems unlikely that one of these sites is representative of the proposed non-specific NTP binding site, which the preceding data suggests is much weaker binding than the catalytic site. One possible explanation for the two-site fit involves the different conformers of Mg$^{2+}$-coordinated ATP, namely the “open” conformer, in which the metal ion only coordinates the phosphates, and the “macrochelated” conformer, in which the metal ion is coordinated to the phosphate and the base. Interestingly, the macrochelated form represents ~10% of the ATP population at physiological salt conditions. Thus, this conformer could represent the
Figure 3.6 ITC titration curves for NTPs binding to K296R. (A) GTP (green trace), UTP (blue trace), and CTP (orange trace) binding to K296R. Titration of each NTP into buffer is included (black traces). NTP traces are offset from buffer traces by ~0.08 µcal/sec in raw data (upper panels) for clarity. Legends are provided in plots. (B) K296R binding to ATP (red trace). Titration of ATP into buffer is included (black trace). Two aberrant data points at ~40 min were omitted from the integrated data (lower panel). ATP titration curve is fitted to a two site binding model as per Equation 3.3, and thermodynamic parameters are provided to the right of the plot. Legend is provided in the plot.
“site 2” observed by ITC titration.

In sum, the ITC data provide further support for the high specificity and affinity of the catalytic ATP binding site. No significant interaction was observed for GTP, CTP, and UTP binding to K296R, suggesting the putative 5′-ppp binding site is very weak binding, although it is also possible that binding to this 5′-ppp site is simply thermoneutral (ΔH ~ 0), and thus not detectable by ITC.

3.4.6 Kinetics of NTP binding to K296R

Stopped-flow fluorescence measurements were determined to further characterize the nature of NTP binding by PKR and, by inference, of PKR activation by 5′-ppp-ssRNA. The kinetics of mant-ATP association and dissociation were previously characterized by Cole and coworkers by fluorescence stopped-flow whereby excitation at 295 nm results in FRET of tryptophan to mant-ATP. We repeated the dissociation (pulse-chase) experiments with prebound PKR•mant-ATP (pulse) in which ATP is in excess (chase) and the slow step is determined by the off-rate of mant-ATP, as per Scheme 3.1, where PKR•mant-ATP′ ⇌ PKR•mant-ATP represents a conformational change:

\[
\text{PKR} \cdot \text{mant-ATP}' \rightleftharpoons \text{PKR} \cdot \text{mant-ATP} \xrightarrow{\text{slow}} \text{PKR} + \text{mant-ATP} \xrightarrow{\text{fast}} \text{PKR} \cdot \text{ATP}
\]

We again utilized K296R and observe monophasic kinetics with a \( k_{\text{obs}} \) of 17.9 ± 0.3 s\(^{-1}\) (Figure 3.7.A). On the other hand, this rate is in agreement with a reported rate for mant-ATP dissociation of 18.6 ± 0.1 s\(^{-1}\). However, the previously reported dissociation of mant-ATP by ATP competition was biphasic, with a \( k_{\text{obs}} \) of 134 s\(^{-1}\) for the
Figure 3.7 Kinetics of mant-ATP dissociation from K296R measured by fluorescence-detected stopped-flow. Mant-ATP was excited at 295 nm and changes in fluorescence upon competition with ATP were monitored using a 450 nm bandpass filter. (A) In the presence of 1 µM K296R, 40 µM mant-ATP was competed with 2 mM ATP. (B) In the presence of 1 µM K296R, 40 µM mant-ATP was competed with 4 mM ATP. (C) In the presence of 1 µM K296R, 100 µM mant-ATP was competed with 2 mM ATP. For (A), (B), and (C), each data set represents the average of 6-8 shots, and black lines represent single-exponential fits to the data. Rate constants ($k_{obs}$) and amplitudes (A) are provided.

For reasons that are unclear, this fast phase was not apparent in our hands.

As a control, we increased the concentration of ATP chase from 2 to 4 mM. As shown in Figure 3.7.B, increasing the concentration of competitor ATP from 2 mM to 4 mM has
essentially no effect on the rate ($k_{\text{obs}} = 16.7 \pm 0.6 \text{ s}^{-1}$). This confirms the mechanism in Scheme 3.1 in which the observed rate for mant-ATP dissociation is not dependent upon ATP concentration (Figure 3.7.B).

Next, we increased the mant-ATP concentration by 2.5-fold (to 100 $\mu$M) in hopes of filling the 5’-triphosphate binding site. Increasing the concentration of mant-ATP to 100 $\mu$M gave a similar $k_{\text{obs}}$ of $14.1 \pm 0.7 \text{ s}^{-1}$ (Figure 3.7.C), and the amplitude decreased by 2-fold relative to 40 $\mu$M mant-ATP (Figure 3.7.A). This decrease in amplitude likely resulted from resetting the high voltage to a lower value due to the increase in signal from the additional mant-ATP. Thus, similar dissociation events involving a similar number of photons would result in a lesser amplitude. It is noted that in all three panels in Figure 3.7, a fast initial phase is observed with an amplitude of ~0.1. This phase is likely unrelated to dissociation from K296R as it is also observed in a control experiment in the absence of K296R (data not shown).

The assay in Figure 3.7.A was repeated with GTP and UTP as competitors (Figure 3.8.A and B). Consistent with the above proposed model, whereby the observed slow dissociation event is attributed to binding in the highly specific catalytic site, dissociation of mant-ATP is not observed in the presence of GTP or UTP in our assay. It is noted that for GTP (Figure 3.8.A, inset) small upward and downward trends were observed at early time points in individual traces, which averaged out.
Figure 3.8 Kinetics of mant-ATP dissociation from K296R upon competition with GTP or UTP, measured by fluorescence-detected stopped-flow. A) In the presence of 1 µM K296R, 40 µM mant-ATP was challenged with 2 mM GTP. (B) In the presence of 1 µM K296R, 40 µM mant-ATP was challenged with 2 mM UTP. For both (A) and (B), experiments were conducted as described in Figure 3.7. It is noted that the amplitude of the noise for the individual traces (~0.1) and especially in the averaged traces (~0.05) is comparable to the noise in the average ATP trace (~0.05) from Figure 3.7.

3.5 Conclusions

The results presented herein provide insight into the mechanism of 5’-triphosphate-dependent activation of PKR by ssRNAs with short stem loops. We have shown previously that PKR activation by these ssRNAs is highly specific for the triphosphate moiity, as 5’-OH, 5’-p, 5’-pp, and 5’-7mG were not sufficient to promote activation in vitro, and these results were reproducible in vivo. PKR activation by ssRNA is surprising in light of the prevailing mechanism for RNA-dependent activation, whereby
dsRNA of sufficient length serves to promote functional dimerization of PKR. The 5’-ppp-ssRNAs characterized in our earlier study contain minimal secondary structure, suggesting PKR operates by an alternative mechanisms. We thus sought to define the interaction of PKR and 5’-ppp-ssRNA by characterizing the 5’-ppp binding pocket within PKR using a variety of functional and biophysical assays.

Two possible models for PKR binding the 5’-ppp of ssRNA activators are the “1-site” model, in which the catalytic binding cleft in the kinase domain binds both ATP for catalysis and the 5’-ppp of ssRNA, and the “2-site” model, in which the 5’-ppp binding site is separate from the catalytic ATP site, located either in the kinase domain or in the dsRBD. The data provided herein support the 2-site model, in which the catalytic ATP binding site (referred to as “site 1”) is highly specific for ATP, and the 5’-ppp binding site (“site 2”) is more permissive and subsequently weaker binding. This specificity is qualitatively summarized in Table 3.1 in which functional assays utilized activating ss-RNA-47s starting with 5’-pppG, -pppA, -pppC, and –pppU, as well as the four NTPs and various other NTP functional groups to probe the specificity of the two sites. Consistent with the notion of a highly specific catalytic site, only ATP served as an effective

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>GTP</th>
<th>CTP</th>
<th>UTP</th>
<th>ppp</th>
<th>pp</th>
<th>GMP</th>
<th>GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>–</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Site 2</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>–</td>
<td>N</td>
<td>Y</td>
</tr>
</tbody>
</table>

Table 3.1 Summary of functional assay results described in Figures 3.2-3.4 as applied to the 2-site model of NTP binding to PKR, where “Site 1” refers to the catalytic ATP binding site, and “Site 2” refers to the 5’-ppp binding site.
phosphoryl donor and as a competitor for PKR activation by traditional dsRNA that does not require a 5’-ppp. The selectivity at site 1 of PKR for ATP makes sense from a biological standpoint, because hydrolysis of ATP and GTP is energetically comparable. Thus, binding specificity likely serves as a selective pressure to avoid catalysis by GTP, which could have detrimental consequences for PKR regulation in the cell. Similarly, the permissivity of the proposed site 2 allows for PKR activation by 5’-ppp activators that start with any of the four nucleotides. Indeed, most human viral RNAs begin with G or A, and so ability of PKR to recognize the 5’-ppp of viral RNA starting with different nucleotides could be crucial to mounting an early immune response.

In addition to differences in NTP specificity, binding affinities of the two sites also varied, as summarized in Table 3.2. In concert with higher specificity at site 1,

<table>
<thead>
<tr>
<th>Method</th>
<th>NTP</th>
<th>$K_D$</th>
<th>$T$ (°C)</th>
<th>PKR form</th>
<th>RNA?</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKR activation competition</td>
<td>ATP</td>
<td>157 µM</td>
<td>30</td>
<td>HO-PKR</td>
<td>Y (79 bp)</td>
<td>1</td>
</tr>
<tr>
<td>PKR activation competition</td>
<td>ATP</td>
<td>280 µM</td>
<td>30</td>
<td>HO-PKR</td>
<td>Y (ppp-ssRNA)</td>
<td>2</td>
</tr>
<tr>
<td>Fluorescence competition</td>
<td>ATP</td>
<td>~47 µM</td>
<td>~22</td>
<td>K296R</td>
<td>N</td>
<td>1</td>
</tr>
<tr>
<td>Fluorescence competition</td>
<td>GTP</td>
<td>~500 µM</td>
<td>~22</td>
<td>K296R</td>
<td>N</td>
<td>2</td>
</tr>
<tr>
<td>ITC</td>
<td>ATP</td>
<td>4 µM (n=1.3), 19 µM (n=0.1)</td>
<td>30</td>
<td>K296R</td>
<td>N</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3.2 Summary of binding affinities determined by functional and biophysical assays described in Figures 3.3 and 3.5-3.6.
tighter binding is also observed. Indeed, thermodynamic parameters for the proposed site 2 could not be determined by ITC due potentially to the weakness of this interaction. Additionally, kinetic analysis suggests that while binding to the catalytic ATP binding site is occurs on the order of seconds, binding to the second site may be more transient. Repetition of these assays with 5’-ppp-RNA as the chase could provide more direct information of the rate of 5’-ppp binding, as the addition of the RNA chain may stabilize the interaction.

Overall these data suggest that the 5’-ppp binding site may be more diffuse and electrostatic in nature as opposed to a tight binding pocket. This notion is consistent with negative results from a BLAST search on PKR, which did not reveal strong matches to triphosphate-binding proteins (data not shown); sequence homology was only observed between other dsRBD-containing and other eIF2α kinases. The recently solved crystal structure of another 5’-ppp sensing innate immune protein, RIG-I, bound to RNA with a 5’-ppG revealed a basic patch that binds to the phosphates through electrostatic contacts to lysines and stacking interactions with a surrounding phenylalanine.27 A similar binding site may exist within PKR, possibly within the patch of mixed acidic and basic residues in the linker region between the dsRBD and the kinase domain (see Chapter 1, Figure 1.4), as some of the contacts to the 5’-ppp may be mediated by triphosphate-coordinated Mg$^{2+}$ ions.

Unsuccessful attempts were made to crosslink a photoreactive nucleotide analog, ppp-8-azidoATP, to PKR to identify the location of the 5’-triphosphate binding site. Further crosslinking studies are in progress that involve incorporation of 8-azidoATP into
RNA transcripts both at the 5’-ppp position and within the short stem-loop region for use in crosslinking. These experiments could provide insight into the structure-function relationship of PKR and the 5’-ppp and could provide further mechanistic information regarding the novel activators by revealing whether the dsRBD is in fact involved in recognition of the short stem-loops.

3.6 Acknowledgements

We thank Marc Dreyfus at Laboratoire de Genetique Moleculaire for the generous gift of ENS0134T BL21 cells containing plasmid encoding T7 RNAP with P266L mutation. We also thank Joshua Sokoloski for helpful discussions.

3.7 References


Chapter 4

Regulation of PKR by HCV IRES RNA: Importance of domain II and NS5A

[Published, as a paper entitled “Regulation of PKR by HCV IRES RNA: Importance of Domain II and NS5A” by Rebecca Toroney, Subba Rao Nallagatla, Joshua A. Boyer, Craig E. Cameron, and Philip C. Bevilacqua in Journal of Molecular Biology 2010 400: 393-412.]

4.1 Abstract

Protein kinase R (PKR) is an essential component of the innate immune response. In the presence of double-stranded RNA (dsRNA), PKR is autophosphorylated, which enables it to phosphorylate its substrate, eukaryotic initiation factor 2α, leading to translation cessation. Typical activators of PKR are long dsRNAs produced during viral infection, although certain other RNAs can also activate. A recent study indicated that full-length internal ribosome entry site (IRES), present in the 5’-untranslated region of hepatitis C virus (HCV) RNA, inhibits PKR, while another showed that it activates. We show here that both activation and inhibition by full-length IRES are possible. The HCV IRES has a complex secondary structure comprising four domains. While it has been demonstrated that domains III–IV activate PKR, we report here that domain II of the IRES also potently activates. Structure mapping and mutational analysis of domain II indicate that while the double-stranded regions of the RNA are important for activation, loop regions contribute as well. Structural comparison reveals that domain II has multiple, non-
Watson–Crick features that mimic A-form dsRNA. The canonical and noncanonical features of domain II cumulate to a total of ~33 unbranched base pairs, the minimum length of dsRNA required for PKR activation. These results provide further insight into the structural basis of PKR activation by a diverse array of RNA structural motifs that deviate from the long helical stretches found in traditional PKR activators. Activation of PKR by domain II of the HCV IRES has implications for the innate immune response when the other domains of the IRES may be inaccessible. We also study the ability of the HCV nonstructural protein 5A (NS5A) to bind various domains of the IRES and alter activation. A model is presented for how domain II of the IRES and NS5A operate to control host and viral translation during HCV infection.

4.2 Introduction

Protein kinase R, (PKR) is an integral part of the human innate immune response mechanism, which is the cell’s first line of defense against viral infection.\(^1\,^2\) Upregulated by interferon induction (IFN), PKR becomes activated by binding long stretches of double-stranded RNA (dsRNA) (≥ 33 bp), leading to homodimerization and autophosphorylation.\(^3\,-\,^5\) The C-terminal kinase domain of phosphorylated PKR then phosphorylates its substrate, eukaryotic initiation factor 2α (eIF2α), leading to shutdown of cellular translation and inhibition of viral proliferation.\(^6\) One function of PKR as part of innate immunity is to discriminate between self and non-self, which it accomplishes by recognition of molecular patterns in RNA.\(^7\) This recognition is mediated in large part by two tandem N-terminal dsRNA binding motifs (dsRBMs) known as the dsRNA binding
domain (dsRBD), which sense RNA in a non-sequence-specific fashion through contacts to the phosphate backbone in the major groove and 2'-hydroxyls in the minor groove.\textsuperscript{8–10}

It has been clearly established that PKR recognizes and is regulated by viral RNAs.\textsuperscript{6} During viral infection, PKR is thought to be primarily activated by viral genomic dsRNA, although transient dsRNA from replicative intermediates of RNA viruses or transcription intermediates of DNA viruses can also activate PKR.\textsuperscript{6} Typically, viral RNAs activate PKR through long contiguously base-paired regions, a feature that is rare in cellular RNAs. However, RNAs with more complex secondary structures also activate PKR, mimicking dsRNA via RNA dimerization or coaxial stacking of shorter helices.\textsuperscript{11–13} Some of these RNAs also contain certain helical imperfections, such as bulges and internal loops.\textsuperscript{7,14} On the other hand, certain viruses, such as Epstein–Barr and adenovirus, produce noncoding RNAs with multi-helix junctions that function to inhibit PKR activity.\textsuperscript{15–17} In this study we seek to elucidate the structural basis of PKR regulation by a particularly complex noncoding viral regulator of PKR, the internal ribosome entry site (IRES) of the hepatitis C virus (HCV). We also illustrate the importance of non-Watson–Crick secondary structure and structural mimicry in PKR recognition of HCV IRES RNA.

Hepatitis C is a single-stranded positive-sense RNA virus that encodes a single polyprotein flanked by 5’- and 3’-untranslated regions (UTRs).\textsuperscript{18,19} The IRES element, located in the 5’-UTR, functions in recruiting and positioning translation initiation complexes and the ribosome at the start codon.\textsuperscript{20,21} The secondary structure of the HCV IRES is highly conserved, consisting of four distinct independently folded domains that form an overall extended and innately flexible structure (Figure 4.1).\textsuperscript{22–25} These domains
Figure 4.1 Secondary structure of 5’-end of HCV RNA. Shown are nucleotides 1-388, which we refer to as ‘full-length HCV IRES RNA’. Domains are numbered in Roman numerals, with domain II boxed. Translation start codon is boxed (domain IV). Inset: Secondary structure of domain II construct employed and determined in this study containing residues 39-119, with the two G nucleotides added at the 5’-end to aid in transcription priming in boldface. Pairing (P1, P2…) and loop regions (L1, L2…) in domain II are labeled. Asterisk downstream of domain II indicates the 3’-end of domains I–II (1-130) construct and the 5’-end of domains III–IV (131-388) construct. Domain III is further labeled according to its subdomains IIIa-IIIf.
perform essential functions during viral replication and translation: domains I and II are indispensable for genome replication, while domains II, III, and IV are critical for translation initiation.\textsuperscript{26-28} In particular, cryo-electron microscopy studies indicate that domain II and elements of domain III make direct contacts with the 80S ribosome.\textsuperscript{29,30} Domain II induces conformational changes in the 40S subunit, potentially mediating the joining of the 40S and 60S subunits, while domain III positions the start codon, located in domain IV, within the mRNA binding site. Domain II is of particular interest as it is the only domain that functions in both replication and translation. Overall, the HCV IRES represents a highly structured, non-self RNA with domains that serve disparate roles within the virus life cycle, suggesting that it may represent an ideal target for RNA sensors in the innate immune system. Indeed, previous studies show that full-length IRES can inhibit\textsuperscript{31} or activate PKR\textsuperscript{32}, with the latter study assigning this function to domains III–IV.

Herein we analyze full-length HCV IRES from HCV-1b and its isolated domains as regulators of PKR’s phosphorylation activity. Our results indicate that full-length IRES can serve both as an activator and as an inhibitor of PKR, with the particular function depending on the concentration of the IRES. Surprisingly, we find that while a construct consisting of domains III–IV does activate PKR as previously reported,\textsuperscript{32} domain II, which contains fewer base pairs and is interrupted by multiple imperfections, is the more potent activator. We employ footprinting and mutational analysis to map the interaction of PKR within domain II and observe that the apical and internal loops of domain II are particularly important. Overlays with reference model RNA structures suggest that the structural features of these regions of domain II may in fact mimic
dsRNA. Lastly, we investigate the role of HCV nonstructural protein 5A (NS5A) as an antagonist of PKR activation and present an overall model of PKR function that accommodates these data.

4.3 Materials and Methods

4.3.1 RNA sequences

The 79 bp (dsRNA-79) control was prepared by transcribing opposing strands of pUC19 and annealing as previously described. The remaining RNAs were prepared by \textit{in vitro} transcription from a linearized plasmid, a double-stranded PCR product, or a hemi-duplex DNA template (see below). Following are the sequences of RNA used in this study.

**HCV truncations**

1-388 (full-length IRES):
5’GCCAGCCCCCGAUUGGGGGCGACACUCCACCAUAGAUCACUCCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAGCGUCUAGCCAUGGCGUUAGUAUGAGUGUCGUGCAGCCUCCACGAGCCCCCCUCGCCGAGAGCCCAGGACGACGACCGGGGUCCUUUCUUUGGAUCAACCCGCUCAGCCUGAGAUUUUGGGCGUGCCCCCCCGAGACGACGCUAAGCAGUGUUGGUGGCGCAAAGGCUUUGGUGUACUGCCUAGGAUGGUAGCUUGCPAGUCUCGUGCCGAAGGCGCGCCGAACUAGCGACGACGUAACCUCAGCGUAGCCACGCACCCCGUAGGUGUUCGGCCAGCCGC

1-130 (domains I–II):
5’GCCAGCCCCCGAUUGGGGGCGACACUCCACCAUAGAUCACUCCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAGCGUCUAGCCAUGGCGUUAGUAUGAGUGUCGUGCAGCCUCCACGAGCCCCCCUCGCCGAGAGCCCAGGACGACGACCGGGGUCCUUUCUUUGGAUCAACCCGCUCAGCCUGAGAUUUUGGGCGUGCCCCCCCGAGACGACGCUAAGCAGUGUUGGUGGCGCAAAGGCUUUGGUGUACUGCCUAGGAUGGUAGCUUGCPAGUCUCGUGCCGAAGGCGCGCCGAACUAGCGACGACGUAACCUCAGCGUAGCCACGCACCCCGUAGGUGUUCGGCCAGCCGC
131-388 (domains III–IV):
5’GGGAGAGCCAUAGUGGUCUGCAGGAACCCGUGAGUACACCGGAAUUGCCA
GGACGACCGGUGCUCUUCCUUGGAUCAACCCGCUCAAUUGCCUGAGAUUUUG
GGCGUGCCCGCCCGAGACUGCUAGCGAGUAGUUGUGUGUCGCGAAAGGC
CUUGUGGUACUGCCUGAUAGGGCUUGCGAGUGCGCCCCGGAGGAGUCUCGU
AGACCGUGCAACAUAGAGCAGAAUCCUAACCUCUAAAGAAAAACAAAGG
GCGCAGCCGC

39-119 (domain II) (two additional G’s at the 5’-end included to prime transcription are in boldface):
5’GGACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAAGCGUCUAGC
CAUGGCGUUAUGAGUGUGCUGCAGCCUCCAGGA

**Domain II mutants** (additions or substitutions are indicated by underlines, and deletions are indicated by dashes):

**L4–U5:**
5’GGACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAAGCGUCUUUUU
UUUGGCGUUAUGAGUGUGCUGCAGCCUCCAGGA

**Δ2bp:** 5’GGACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAAGCGUCUAGC
CCAUGGCGUUAUGAGUGUGCUGCAGCCUCCAGGA

**+2bp:**
5’GGACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAAGCGUCUAGC
CCAUGGCGUUAUGAGUGUGCUGCAGCCUCCAGGA

**G71A/G94A:**
5’GGACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAAGCGUCUAGC
AGCCAUGGCGUUAUGAGUGUGCUGCAGCCUCCAGGA

**C104U:**
5’GGACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAAGCGUCUAGC
CAUGGCGUUAUGAGUGUGCUGCAGCCUCCAGGA

**ΔL1:** 5’ GGACUCCCCUGUGAGG——CUUGUUCACGCAGAAAAGCGUCUAGCC
AUGGCGUUAUGAGUGUGCUGCAGCCUCCAGGA

**PCR primers** (T7 promoter is underlined, additional 5’-end G’s are in boldface):

1-130 TS: 5’GAAATTAATACGACTCACTATAGCC
1-130 BS: 5’GGGAGGGGGGTCTCTGGA
131-388 TS: 5’GAAATTAATACGACTCACTATAAGGGAGAGCCATAGTGTTCTG
131-388 BS: 5’GCAGCGCGCCCTTTGG
39-119 TS: 5’GAAATTAATACGACTCACTATAAGGACTCCCTGTGAGGAAC
39-119 BS: 5’TCCTGGAGGCTGCACGAC

4.3.2 Protein preparation

Full-length PKR and the dsRBD (p20) containing N-terminal His$_6$ tags were purified from *Escherichia coli* BL21(DE3) Rosetta cells (Novagen) as described elsewhere.$^{14,33}$ Because full-length PKR was isolated in phosphorylated form, the protein was dephosphorylated prior to activation assays by treatment with $\lambda$-phosphatase (NEB).$^{14,34}$ His-$\Delta$-NS5A, which contains amino acids 2005-2419 of the HCV polyprotein, was prepared as previously described.$^{35}$ This construct has the 32 amino acid, N-terminal membrane-anchor domain deleted.

4.3.3 RNA preparation

Full-length HCV IRES (1-388) was transcribed by combining 1.5 $\mu$g of linearized pUC18 plasmid containing a T7 promoter$^{36}$ with 1.8 $\mu$g of T7 RNA polymerase, along with 40 mM Tris (pH 8), 33 mM Mg(OAc)$_2$, 40 mM DTT, 2 mM spermidine, and 7 mM of each NTP and incubating at 37 °C. After 3.5-4 h, the reaction was quenched by adding 95% (v/v) formamide loading buffer. RNA was then purified by fractionating on a polyacrylamide denaturing gel (7 M urea, 1X TBE). The transcript was identified by UV
shadowing, excised from the gel, and eluted overnight at 4 °C in 1X TEN$_{250}$. The RNA was then ethanol precipitated and resuspended in 1X TE buffer (pH 7.5) and stored at -20 °C. RNA concentration was determined spectrophotometrically.

Transcription templates for HCV 1-130, 39-119 and 131-388 were prepared by PCR amplification of the target sequence from the pUC18 plasmid using appropriate TS and BS DNA primers. Templates for each RNA included a T7 promoter sequence at the 5’-end. Additionally, one to two G’s were included at the start of the RNA sequence in order to promote transcription. RNA was transcribed directly from the PCR products using Ambion Inc. T7 transcription kits and purified as described above. HCV 39-119 mutants were prepared by transcription from a hemi-duplex DNA template (IDT) with a T7 promoter$^{37}$ using Ambion T7 kits and purified. Prior to each experiment, RNAs were thawed and then renatured by heating to 90 °C for 3 min, followed by slow cooling to room temperature for 10 min.

### 4.3.4 5’-End-labeling of RNA

For use in gel-shift and structuring mapping/footprinting assays, RNAs were 5’-end-labeled with $^{32}$P. In order to remove the 5’-triphosphate, we first treated RNA with CIP, as previously described.$^{7}$ The RNA was 5’-end-labeled with PNK (NEB) and again purified by gel electrophoresis, excised, eluted overnight at 4 °C, and ethanol precipitated. The RNAs were resuspended in 1X TE, and the concentration was determined using a liquid scintillation counter (Beckman).
4.3.5 PKR activation and inhibition assays

RNAs were tested for their ability to activate PKR autophosphorylation and eIF2α phosphorylation. PKR was first treated with λ phosphatase (NEB) at 30 ºC for 1 h followed by addition of the phosphatase inhibitor, sodium orthovanadate. Next, 0.6 µM dephosphorylated PKR was incubated with RNA at various concentrations in 20 mM HEPES (pH 7.5), 4 mM MgCl₂, 100 mM KCl, 100 µM ATP (Ambion), 1.5 mM DTT, and 1.5 µCi [γ³²P]-ATP for 10 min at 30 ºC. In certain cases, 3 µM eIF2α (10-fold excess over PKR) was added and incubated for an additional 10 min. In PKR inhibition studies by HCV IRES RNA, poly I:C (Sigma) of approximate size 30-200 bp was used as an activator of PKR. HCV IRES RNA and poly I:C were added prior to dephosphorylated PKR and reaction components. All reactions were quenched by addition of SDS loading buffer and then loaded on 10% SDS-PAGE gels. Gels were dried and exposed to a storage PhosphorImager screen and then scanned on a Typhoon PhosphorImager and quantified using ImageQuant (Molecular Dynamics). In all experiments, a negative control containing TEK₁₀₀ instead of RNA and a positive control containing 0.01 or 0.1 µM 79 bp dsRNA were included. Data were normalized after subtracting a background value averaged from different portions of the gel.

4.3.6 Native mobility gel-shift assays

To determine the binding affinity of the dsRBD of PKR (p20) or NS5A for certain RNAs, we incubated excess p20 or NS5A (0.05-5 µM) and trace amounts of 5’-³²P-end-labeled RNA (~ 2 nM) with 1 mg/mL herring sperm DNA, 10 mM NaCl, 25 mM HEPES (pH 7.5), 5 mM DTT, 0.1 mM EDTA, 5% glycerol, 0.1 mg/mL bovine serum
albumin, and 0.01 % NP-40 for 30 min at 22 °C. 33 Samples were loaded onto 0.5X TBE native gels (29:1 crosslink) at 16 °C while the gel was running, and fractionated for 2-2.5 h. Gels were dried and exposed to storage PhosphorImager screens overnight.

4.3.7 Enzymatic structure mapping

Trace amounts of 5’-32P-end-labeled RNAs were digested in the presence of single-strand-specific (RNase T1, RNase A) or double-strand-specific (RNase V1) nucleases under native conditions (20 mM HEPES, pH 7, 100 mM NaCl, and 4 mM MgCl2) for 15 min at 37 °C. To generate the T1 ladder, we incubated labeled RNA under denaturing conditions in 0.01 U/µL T1, 18 mM Na-citrate (pH 3.5), 0.9 mM EDTA, and 6 M urea for 30 min at 50 °C. 13 For the hydrolysis ladder, labeled RNA was incubated in 100 mM Na2CO3/NaHCO3 and 2 mM EDTA for 4 min at 90 °C. All reactions were immediately quenched by addition of an equal volume of 0.2 M EDTA/formamide/0.2% SDS loading buffer and boiled before fractionating on a 12% polyacrylamide, 8.3 M urea sequencing gel. Nuclease concentrations that gave limited digestion were as follows: 0.01 U/µL RNase T1 (Ambion), 1 ng/mL RNase A (Ambion), and 1.6 U/µL RNase V1 (Pierce).

4.3.8 Nuclease footprinting

5’-32P-end-labeled RNAs were treated under the same native conditions as in structure-mapping experiments, with the following additions: 130 ng tRNA^Phe, 1.5 µM BSA, and 10 µM p20. Prior to treatment with nucleases, the samples were incubated at 22 °C for 30 min to allow the RNA and p20 to bind. Appropriate nucleases were then
added at the same concentrations as in the structure-mapping experiments. Digestions for RNase A and RNase V1 proceeded at 37 °C for 15 min and 22 °C for 30 min, respectively. Samples were quenched with an equal volume of 0.2 M EDTA/formamide/0.2% SDS loading buffer and loaded on a 12% polyacrylamide, 8.3 M urea sequencing gel.

4.3.9 In-line probing

In-line probing experiments rely on self-cleavage of the RNA at slightly elevated pH by in-line attack of the 2’-hydroxyl. Patterns of cleavage often change in the presence of proteins as well. 5’-32P-end-labeled RNAs were mixed with the following components: 50 mM Tris (pH 8.3), 20 mM MgCl₂, 100 mM KCl, and p20 (0.625, 1.25, 2.5, 5, or 10 µM). The samples were then incubated at 27 °C for 40 h, followed by addition of an equal volume of formamide/1.5 mM EDTA/10 M urea loading buffer. Loading buffer was also added to an additional sample of labeled RNA that was not subjected to reaction. Samples were then fractionated on a denaturing (8.3 M urea) 12% gel, along with T1 and hydrolysis ladders, as per structure-mapping and footprinting experiments. The gel was analyzed on ImageQuant by drawing a line through each of the lanes and plotting the raw counts.

4.3.10 Inhibition of PKR activation by NS5A

Following is the general approach taken for studies on inhibition of PKR by NS5A, although changes to the order of addition were also explored, as described in the legend to Figure S5. A final concentration of 2.5 µM domain II or domains III–IV, or a
final concentration of 0.1 µM 79 bp RNA, was pre-incubated with various concentrations of NS5A (stored in protein storage buffer: 50 mM HEPES pH 7.5, 50 mM NaCl, 0.1% NP-40, and 1 mM DTT) for 10 min at 30 ºC. In the 0 µM NS5A lanes, an equivalent volume of protein storage buffer was added to keep buffer concentrations consistent across lanes. Dephosphorylated PKR and activation assay reaction components (see above) were then added and incubated at 30 ºC for an additional 10 min. Reactions were quenched and analyzed as per PKR activation assays (see above).

4.3.11 Structural comparisons

RMSD calculations between domain II and 19 bp A-form helix were conducted as follows. Initially, visual inspection was carried out in PyMOL\textsuperscript{40} where the structural similarities between an A-form helix crystal structure (PDB ID: 1QC0) and the domain II lowest-energy NMR structure (PDB ID: 1P5O) became apparent. RMSDs were calculated in VMD version 1.8.7\textsuperscript{41} using a selection script and the measuring utility. The sugar-phosphate backbone of domain II nucleotides 59-73, 95-105, and 107-109 were fit to A-form nucleotides 1-15, 24-34, and 36-38, respectively. Additionally, the S-turn of domain II was accommodated by fitting the hydroxyl oxygens of domain II nucleotides 90-93 to nucleotides 19-17 and 23 of the A-form helix. RMSD calculations between the apical loops of domain II average energy NMR structure (PDB ID: 1P5P) and snR47h (PDB ID: 1T4L) were conducted in a similar fashion.
4.4 Results

4.4.1 PKR can be both activated and inhibited by full-length HCV IRES

An early study\(^{31}\) indicated that the HCV IRES functions similarly to viral RNA from Epstein–Barr\(^{15}\) and adenovirus\(^{17,42}\) in that it inhibits PKR activation. Typically, such inhibition occurs through competition between viral RNA and dsRNA for binding to the dsRBD. While activating dsRNA promotes dimerization of PKR on a single RNA molecule, inhibitor RNA binds PKR in a manner that prevents functional dimerization, locking PKR in an inactive complex.\(^5\) In this earlier study,\(^{31}\) activation assays were conducted at HCV IRES RNA concentrations below ~80 nM, which turns out to be too low to promote functional dimerization and activation of PKR (see below). A more recent study has shown that at IRES concentrations above ~50 nM, PKR undergoes activation.\(^{32}\) Thus, one prior study showed that the full-length IRES inhibits PKR, while another showed that it activates PKR.

In an effort to reconcile these studies, we first tested full-length HCV IRES (nucleotides 1-388) for inhibition of PKR (Figure 4.2.A). Dephosphorylated PKR (0.6 µM) and polyinosinic:polycytidylic acid (poly I:C) (1 µg/mL), a known activator of PKR, were incubated with increasing concentrations of HCV IRES (Figure 4.2.A).\(^*\) As expected from the earlier study,\(^{31}\) inhibition was observed. Inhibition of poly I:C-mediated activation of PKR was observed starting at 1.25 µM HCV IRES, with inhibition increasing with IRES concentration. The slight increase in activation with

\(^*\) In the inhibition study, the concentration of IRES is kept at or above 10 µg/mL, while the concentration of poly I:C is constant at 1 µg/mL. This set up assures at least 10 times more nucleotides of the inhibitor.
lower concentrations of HCV IRES (Figure 4.2.A) suggested that the IRES might also function as an activator.

**Figure 4.2** Inhibition and activation of PKR by full-length HCV IRES RNA (1-388). (A) Inhibition of poly I:C-mediated activation of PKR by HCV IRES. The amount of poly I:C in all lanes was 1 µg/mL, and concentrations of HCV IRES were 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5 and 10 µM. No-RNA/no-poly I:C and no-HCV IRES lanes are included. Phosphorylation activities are provided under the gel and were normalized relative to the lane in which only poly I:C was present. (B) Activation of PKR by HCV IRES. RNA concentrations were 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, and 5 µM. A no-RNA lane is included. Phosphorylation activities were normalized to the most reactive lane, 0.64 µM IRES. For both (A) and (B), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated, and graphical representations of phosphorylation activities as a function of full-length IRES concentration are provided.
Next, we tested full-length HCV IRES for activation of PKR in the absence of poly I:C. Activation was tested over full-length IRES concentrations ranging from 0.02 to 5 µM (Figure 4.2.B). We confirmed that full-length HCV IRES activates PKR, starting at ~160 nM IRES. As in the presence of perfect dsRNA, PKR activation displays a bell-shaped dependence on HCV IRES concentration with maximal activation near 0.64 µM HCV IRES. Relative to a 79 bp standard activator, full-length IRES activates up to ~125% the level of activation by 79 bp. We also note that the concentration of HCV IRES required to inhibit PKR is similar in Figures 4.2.A and B, as expected. The inhibitory arm is typical for PKR activators and is attributed to titration of active PKR dimers to monomers on separate RNA molecules. In summary, PKR is both activated and inhibited by the IRES, with the particular activity depending on the reaction conditions.

4.4.2 PKR is activated by multiple IRES domains, but most potently by domain II

The HCV IRES folds into a highly conserved, stable secondary structure with long dsRNA-like stems containing numerous defects (Figure 4.1). Within the IRES are four independently-folding domains, numbered I-IV. These domains include domain I, a GC-rich hairpin; domain II, a stem-loop structure interrupted by two bulges and three internal loops, L1–L3, and an apical loop, L4 (Figure 4.1, inset); domain III, a large structure divided into five subdomains (IIIa–IIId), that include 2 four-way junctions and a three-way junction, numerous internal loops and bulges, and a pseudoknot (Figure 4.1); and domain IV, a small hairpin at the base of an internal loop that contains the start codon.
Given the highly complex secondary structure of the HCV IRES, we sought to determine which IRES domains are responsible for activation of PKR. The RNA constructs tested for activation of PKR consisted of HCV 1-130, which includes domains I and II of the IRES (Figure 4.1, 5’-end to the asterisk); HCV 131-388, which comprises domains III–IV (Figure 4.1, asterisk to 3’-end); and HCV 39-119, which contains just domain II (Figure 4.1, inset). A previous study indicated that domains III–IV of the IRES are critical for PKR activation, but that domain II is dispensable and unable to activate PKR independent of the other IRES elements.\textsuperscript{32} We also find that domains III–IV activate PKR, with activation increasing through 5 μM RNA (Figure 4.3.A and C). However, we also find that domains I–II activate in a similar fashion to domains III–IV (Figure 4.3.A and C). In fact, at 5 μM RNA, domains I–II activate PKR to a greater extent, at ~80% the activation level of the 79 bp standard, than domains III–IV, which activate only up to ~50%. Additionally, over all the RNA concentrations tested, domains I–II display 1.5- to 4.5-fold greater activation than domains III–IV. These observations suggest that in full-length IRES, domains I–II make a major contribution to activation.

Within domain I, and downstream of domain II, there are stretches of multiple G’s and C’s that could potentially lead to multimerization of this RNA and significantly increase the total number of base pairs. In this scenario, it is possible that PKR activation observed for domains I–II is actually due to the presence of an RNA dimer, as was recently found for human immunodeficiency virus transactivation response region (HIV TAR) RNA.\textsuperscript{11} To investigate this possibility, we renatured domains I–II at a high RNA concentration of 20 μM and then analyzed for RNA dimers on a native gel at an RNA
Figure 4.3 Activation of PKR by multiple domains of HCV IRES. (A) PKR activation assays of domains I–II (1-130) (left panel) and III–IV (131-388) (right panel). RNA concentrations for both panels were 0.3, 0.6, 1.3, 2.5, and 5.0 µM. (B) PKR activation assay of domain II (39-119) alone. RNA concentrations were 0.15, 0.3, 0.6, 1.3, 2.5, 5 and 10 µM. For both (A) and (B), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. A no-RNA lane and a 79 bp dsRNA lane are included. Phosphorylation activities are normalized to the 79 bp lane and are noted under the gel. (C) Graphical representation of phosphorylation activities from panels (A) and (B) as a function of RNA concentration.

A concentration of 10 µM. A slower-mobility species was observed on both native and denaturing gels (data not shown) that indicated possible dimer formation.

Domain II alone was prepared to eliminate the possibility of RNA dimers. We chose a construct that begins at 39, which eliminates domain I, and ends at 119, which eliminates the C-rich 3’-end. This 39-119 construct, referred to as ‘domain II’ (Fig. 1,
inset), therefore lacks both GC-rich potential dimerization regions. We also note that our
domain II construct has the same number of base pairs as the native domain II (see
below).† On a native gel, in the presence of high concentration of domain II (10 µM),
only one major species was observed, which migrated at the expected mobility for a
monomer relative to RNA markers, and no species were observed at the expected
mobility for a dimer (data not shown). These observations suggest that domain II exists
as a monomer in solution at or below 10 mM RNA.

Activation assays of domain II were then performed at RNA concentrations
ranging from 0.15 to 10 µM (Figure 4.3.B). Within this concentration range, domain II
potently activated PKR, with maximal activation at 5 µM RNA. Additionally, the level
of activation by domain II at 5 µM was ~1.4-fold greater than the activation for the 79 bp
standard at its maximum and greater than the maximal activation for domains I–II and
domains III–IV. Domain II also displayed the bell-shaped dependence on RNA
concentration typical of PKR activators, with activation decreasing slightly at 10 µM
domain II (Figure 4.3.C).‡ We previously reported that a 5’-triphosphate, which is
naturally at the end of T7 transcripts, can contribute to activation of PKR by certain
largely single-stranded RNAs but not by dsRNAs.§ To test whether the 5’-triphosphate
of the domain II transcript contributes to activation of PKR, we removed the 5’-
triphosphate by treatment with calf intestinal phosphatase (CIP). The CIP-treated RNA
activated PKR to the same levels as untreated domain II (Figure B.1), indicating that this

† Two G residues were inserted upstream of nucleotide 39 to aid with T7 transcription (see Figure 4.1, inset). To
eliminate potential interference with RNA folding, these G’s were separated from the base of domain II by five native
single-stranded nucleotides. As shown below, these nucleotides do not interfere with the fold of the RNA or contribute
to dimerization.
‡ Activation assays were not conducted above 10 µM to ensure that the RNA remained as a monomer.
5’-triphosphate does not contribute to activation of PKR. This result is as expected given that domain II normally is found within the context of the full-length IRES and so does not have a 5’-triphosphate and also supports a contribution of domain II to activation of PKR that is similar to that of a dsRNA (see below). To summarize, domain II is a monomeric RNA that potently activates PKR in the absence of domain I and the 3’-flanking region of domain II. That domain II alone activates PKR is surprising because it has only 24 Watson–Crick base pairs and GU wobbles. This observation suggested the possibility of an important role for non-Watson–Crick motifs in activation, as will be discussed further. Next, we compared eIF2α phosphorylation and PKR binding by the various HCV domains.

4.4.3 PKR activation by IRES elements leads to potent phosphorylation of eIF2α

During viral infection, PKR activation via autophosphorylation leads to downstream regulation of translation initiation through phosphorylation of eIF2α, a cellular substrate of PKR. We thus sought to determine the potential biological relevance of PKR activation by IRES elements. Activation assays were performed in the presence of eIF2α and indicated that all IRES elements facilitate potent phosphorylation of eIF2α (Figure 4.4, lower bands). For instance, 0.625 µM full-length IRES led to 77% PKR activation and 110% eIF2α phosphorylation relative to 79 bp control (Figure 4.4.A). Similarly, domains III–IV phosphorylated eIF2α up to 112% (Figure 4.4.B), while domain II led to 125% eIF2α phosphorylation (Figure 4.4.C). Thus, eIF2α phosphorylation mirrors PKR activation: domain II is a stronger activator, which leads to
Figure 4.4  Activation of PKR and phosphorylation of eIF2α by multiple domains of HCV IRES.  (A) PKR/eIF2α phosphorylation by full-length (1-388) HCV IRES.  For both – and + eIF2α lanes, IRES concentrations were 0.625, 1.25, and 2.5 µM.  (B) PKR/eIF2α phosphorylation by domains III–IV (131-388).  For both – and + eIF2α lanes, domains III–IV concentrations were 0.625, 1.25, 2.5, 5, and 10 µM.  (C) PKR/eIF2α phosphorylation by domain II (39-119).  For both – and + eIF2α lanes, domain II concentrations were 0.625, 1.25, 2.5, 5, and 10 µM.  For all panels, 10% SDS-PAGE gels are presented, and positions of phosphorylated PKR (p-PKR) and phosphorylated eIF2α (p-eIF2α) are indicated.  When present, eIF2α was in 10-fold excess over PKR.  PKR activation for all gels was normalized to the 79 bp (–eIF2α) PKR band in panel (C), and eIF2α phosphorylation for all gels was normalized to the 79 bp (+eIF2α) eIF2α band in panel (C).  These values are provided under the gel.
slightly higher eIF2α phosphorylation as compared to domains III–IV. Notably, both
domain II and full-length IRES lead to comparable levels of eIF2a phosphorylation; for
example, 93% and 110% phosphorylation at 0.625 μM RNA was observed, suggesting
that domain II alone may be nearly as effective as full-length IRES in activating PKR.

4.4.4 Binding affinity of IRES elements for the PKR dsRBD p20

Based on our activation assays, we expected that extent of activation for various
IRES elements might correlate with extent of binding to the dsRBD, p20 (20 kDa dsRNA
binding domain of PKR). Native mobility gel-shift assays of full-length IRES, domains
III–IV, and domain II were therefore performed (Figure B.2). We observe that p20 binds
both full-length IRES and domains III–IV tightly and in multiple complexes, with
complex formation starting at ~0.25 μM p20 for the full-length IRES and at ~0.5 μM for
domains III–IV (Figure B.2.A and B). These similarities suggested that the other
domains in the IRES might not contribute substantially to binding. Indeed, domain II
bound considerably more weakly, with very weak complex formation occurring only at
~2.5 μM p20. Additionally, domain II gave only a single, low-mobility complex, which
required a 20% gel to resolve (Figure B.2.C). Thus, domain II appears to bind p20
considerably more weakly than domains III–IV. Despite relatively poor gel-shifting by
domain II, in-line probing assays revealed protection of domain II by p20 as low as 0.6
μM (see below), supporting dynamic binding that gel shifts resolve only poorly.

Notably, the inhibitory arm for PKR activation by full IRES begins at ~1.2 μM
(Figure 4.2.B), while it begins at >5 μM (Figure 4.3.C) for domains III–IV despite
similar gel shifting by p20. This may be because of tight, nonproductive binding modes
for domains III–IV. In addition, activation by domains III–IV is ~3.3- to 4-fold less potent than that by domain II, despite tighter binding of p20 to domains III–IV. These observations also suggest that perhaps some of the binding modes of domains III–IV are nonproductive, or even inhibitory, for activation, perhaps due to spacing of binding sites (see Discussion). These data further suggest that much of the activation by full-length IRES may be due to domain II alone (see Discussion). Because it contributes significantly to PKR activation, we focused our remaining studies on domain II.

4.4.5 Experimental determination of domain II secondary structure

As mentioned, domain II of the HCV IRES is atypical of PKR activators in that it has fewer than 33 canonical base pairs and contains several loop and bulge imperfections.\textsuperscript{22,24} Structures of domain II have been solved and these reveal an overall bent, or hook-like, shape of the domain, induced by an asymmetric internal loop (L1) (Figure 4.1, inset).\textsuperscript{48-50} NMR studies have identified a number of notable features of domain II: stacking in L1, numerous noncanonical base pairs in L2 and L3, and stacking and hydrogen bonding within L4.\textsuperscript{48} Thus, although domain II contains a number of structural motifs that initially appear atypical of a PKR activator, noncanonical motifs that have the potential to act as mimics of dsRNA are present.

Ribonuclease structure-mapping experiments were performed to test the structure of our domain II construct (Figure 4.5). We used ribonucleases T1 and A, which cleave after single-stranded G and single-stranded C and U, respectively, as well as ribonuclease V1, which cleaves before double-stranded and stacked nucleotides.\textsuperscript{51-53} Ribonuclease T1 cleaved after two G’s present in single-stranded loop regions as well as after G’s in the
Figure 4.5 Ribonuclease structure mapping of domain II. (A) RNA was 5'-end-labeled and subjected to limited nuclease digestion or hydrolysis. Denaturing 12% polyacrylamide gel is shown. Lanes are as follows: C is a control sample (no nuclease),
OH− is a limited alkaline digest, and T1, A, and V1 are limited digests with ribonucleases specific for single-stranded G, single-stranded C and U, and double-stranded regions, respectively. Lanes 2 and 3 were performed under RNA-denaturing conditions (denoted ‘Den.’), while lanes 4 and 5 were performed under RNA-native conditions (denoted ‘Nat.’). T1 digestion was also performed under native conditions (not shown). Nucleotides are denoted to the left of the T1 denaturing lane, and pairing and loop regions of domain II are indicated to the right of the gel. (B) Secondary structure of domain II with positions of cleavage by ribonucleases in panel (A) indicated by symbol. Legend is provided and symbol size is proportional to cleavage intensity.

single-stranded 5’-end. Also, there was absence of T1 cleavage after all but one of the G’s predicted to be involved in base pairing, and this G (G60) is adjacent to a G·U wobble, which may induce breathing of the helix. As expected, RNase A cleaved primarily after C’s and U’s in loop regions, especially at U56 in L1 and U80, C83 and C84 in L4. In addition, RNase A cleavage is largely absent from pairing regions, with the exception of some moderate cleavage near bulges or the ends of helical regions, indicative of breathing. Lastly, RNase A did not cleave appreciably after any of the U’s and C’s in L2, consistent with U·U and U·C base pairs from NMR.

The dsRNA-specific ribonuclease RNase V1 cleaved strongly before nucleotides on the 5’-strand of pairing regions P2, P3, and P4; strongly on both strands of P1; and weakly in the 3’-strand of P3 (Figure 4.5.A and B). Cleavage by RNase V1 was also observed before residues A53 and A54 of L1, which is consistent with NMR structures that show stacking of these nucleotides.48 Thus, according to RNA structure mapping, the overall secondary structure of the domain II construct used herein is largely consistent with predicted and NMR secondary structures.
4.4.6 Mapping of p20 binding to domain II

To further characterize PKR’s interaction with domain II, we conducted nuclease and in-line probing footprinting experiments in the presence of p20. Nuclease footprinting was performed by pre-incubating trace amounts of radiolabeled domain II with 10 µM p20, which, according to Figure S2c (bottom panel), should be saturating, and then treating with either RNase A or RNase V1 (Figure 4.6.A and C). We observed variable extents of p20-dependent protection from nuclease digestion throughout domain II (see Figure B.3.A for quantitation). For example, nucleotides in the 3’-strand of L3 (L3_3’) were 75% protected from RNase A cleavage, while C42, which is located in the 5’-single-stranded tail, was only 7% protected. Similarly, nucleotides in the 5’-strand of P3 (P3_5’) were just 30% protected from RNaseV1 cleavage, while C62 of P2_5’ was 50% protected.

One concern was whether reduction in RNase cleavage activity reflected protection by p20 or was instead simply due to inhibition of ribonuclease activity. However, we note that certain domain II nucleotides are cleaved equally by ribonucleases in the absence and presence of p20. For example, P4_5’ is cleaved by RNase V1 and is not appreciably protected by p20. Likewise, C55 of L1 is cleaved by RNase A similarly in the absence and presence of p20.

Because PKR is known to bind to RNA primarily through interactions with the 2’-hydroxyls, we also performed in-line probing footprinting. Any decreases in cleavage observed could be due to direct interaction of the RNA with p20 or to RNA conformational changes that lead to nonreactive alignments of the 2’-hydroxyls. In-line
Figure 4.6. Footprinting of p20 onto domain II. (A) Ribonuclease footprinting. RNA was 5'-end-labeled and saturating p20 (10 µM) was added in indicated lanes. This was incubated at room temperature for 30 min followed by digestion with indicated ribonucleases under native conditions. Control lanes were not subjected to nuclease digestion. Denaturing 12% polyacrylamide gel is shown. Lanes are labeled as per Figure 5. Nucleotides are denoted in the T1 denaturing lanes, and regions of domain II are
indicated next to sites of cleavage in both the RNase V1 and A digestion lanes. (B) In-line footprinting. Details are as in panel (A) with the following exceptions. RNA was subjected to partial digestion for 40 h at pH 8.3 in the presence of increasing concentrations of p20 (0, 0.625, 1.25, 2.5, 5, and 10 µM). NR denotes no reaction. (C) Secondary structure of domain II, with positions of p20-dependent protection from RNase A, RNase V1, and in-line cleavage indicated. Legend is provided in the figure. (D) Stereoview of averaged domain II NMR structure (PDB ID: 1P5P), with positions of p20-dependent protection of 2’-hydroxyls from RNase A, RNase V1, and in-line cleavage indicated. 2’-Hydroxyls are depicted as spheres and colored as per the legend provided in the figure. In cases where protection from nuclease and in-line cleavage occurred on the same 2’-hydroxyl [see (C)], bases were colored according to nuclease protections for simplicity.

Probing experiments were conducted analogously to nuclease footprinting, in that trace domain II was incubated with 10 µM p20, but incubation was conducted for 40 h at slightly elevated pH (pH 8.3) in the presence of 20 mM MgCl₂ (Figure 4.6.B and C). In-line probing revealed p20-dependent protection at nucleotides between 64 and 80, which includes the 5’-strands of P3, L3, and P4, as well as at nucleotides flanking G94 on L3 (see Figure B.3.B for quantitation).

Next, we mapped the nuclease and in-line cleavage protections onto the average minimized NMR structure of domain II (Figure 4.6.D). This revealed that p20 binds primarily above and below the L1 hinge bulge, while mostly avoiding L1 itself (Figure 4.6.D, yellow). Within the protected regions, p20 interacts with the pairing elements P1-P4 of domain II, as expected. In addition, p20 protects portions of L2, L3, and L4, suggesting that these non-Watson–Crick regions may mimic dsRNA, thereby facilitating binding of p20 (see Discussion).
4.4.7 Effects of mutations to domain II on PKR activation

In order to further explore how domain II activates PKR, we conducted a mutational analysis of domain II. Mutations in the apical loop, L4, and the pairing region, P4, of domain II were initially prepared. In particular, the L4 mutant ‘L4–U5’ exchanges the AGCCA nucleotides of the structured apical loop for U5, which should be largely unstructured, while the P4 mutants, ‘+2bp’ and ‘∆2bp’, add and delete 2 bp within P4, respectively (Figure 4.7.A). We find that the addition of two GC base pairs in +2bp, which lengthens and stabilizes P4, enhances PKR activation relative to WT (Figure 4.7.B and C). For example, at 0.3 µM RNA, activation is 2-fold greater than WT activation. Conversely, deletion of two GU base pairs, which shortens the helix, somewhat diminishes activation, such that ∆2bp does not reach the level of WT maximal activation at even the highest RNA concentration tested (Figure 4.7.B and C). Removing structure from the apical loop, in L4–U5, decreases PKR activation to a similar degree as ∆2bp, consistent with interaction with PKR (Figure 4.7.B and C). Overall, this set of mutants suggests that both double-stranded and loops regions contribute to activation of PKR by domain II.

Next, mutations in the three internal loops of domain II were investigated. In particular, ‘G71A/G94A’ and ‘C104U’ potentially make L3 and L2 less structured, and ‘∆L1’ eliminates the bend in domain II (Figure 4.7.A). As compared to the P4 and L4 mutants, the internal loop mutants had more subtle effects on activation (Figure B.4). In particular, the percent activation of all three mutants at 10 µM RNA was ~95%, as compared to 112% for WT, and at 2.5 µM RNA, activation by all three mutants equaled that of WT. To examine possible effects of these mutants in more detail, we performed
Figure 4.7 Activation of PKR by domain II mutants. (A) Domain II mutants (L4–U5, Δ2bp, +2bp, G71A/G94A, C104U, and ΔL1) superimposed on secondary structure of domain II wild-type (WT). (B) PKR activation assays of P4 and L4 mutants (Δ2bp, +2bp, and L4–U5). RNA concentrations for WT and mutants were 0.16, 0.3, 0.6, 1.5, 2.5, and 3.6 µM. Phosphorylation activities for both gels are normalized to 0.01 µM 79 bp in upper gel and are noted under the gel. (C) Graphical representation of phosphorylation activities from panel (B) as a function of RNA concentration. (D) Time course of PKR activation assays for L1, L2, and L3 mutants (ΔL1, C104U, and G71A/G94A). Mutants and WT RNA were tested at 1.25 and 5 µM as indicated, and assays were conducted for 3, 5, and 10 min at each RNA concentration. Phosphorylation activities for both gels are normalized to 0.01 µM 79 bp in upper gel and are noted under the gel. (E) Graphical representation of phosphorylation activities from panel (D) as a function of time, at RNA concentration of 5 µM. For both (B) and (D), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated.

time trials, in which 1.25 or 5 µM RNA was incubated with PKR for 3, 5, and 10 min (Figure 4.7.D and E). At 1.25 µM RNA, activation by C104U and G71A/G94A was essentially equal to that of WT at every time point, while ΔL1 activation was 2 to 3-fold lower (Figure 4.7.D). At higher concentrations of RNA (5 µM), ΔL1 activation was as much as 4-fold lower than WT, while the other mutants were now 1.7-fold lower (Figure 4.7.D and E). The decrease in activation for ΔL1 suggests that the 5-nucleotide bulge may play a role in positioning the PKR dimer on domain II. The smaller decrease in activation for the L2 and L3 mutants suggests that these regions may also help position PKR for activation, although the size, rather than the sequence, of the loop may be the dominating factor. In summary, activation assays on the six RNA mutants support interaction of much of domain II with PKR, including noncanonical regions, consistent with the above footprinting experiments.
4.4.8 Effect of NS5A on PKR activation by HCV IRES domains

Activation of PKR in HCV-infected cells is antagonized by interaction with NS5A, an HCV nonstructural protein.\textsuperscript{54-56} NS5A is an RNA-binding protein that prefers single-stranded GU-rich segments of the 3’-UTR of HCV.\textsuperscript{57} We sought to elucidate a possible connection between NS5A binding and regulation of PKR by HCV IRES RNA.

Inspection of HCV IRES sequence reveals several GU-rich segments, which are present within domains III and IV (Figure 4.1).\textsuperscript{57} Notably, there are no stretches of GU elements in domain II. We performed native gel-mobility shift assays of NS5A with domain II, domains III–IV, and full-length IRES, as well as 79 bp control (Figure 4.8). We find that domains III–IV and full-length IRES bind NS5A tightly, forming multiple complexes with an apparent $K_d$ of $\sim 0.13$ µM, based on loss of approximately half of the unbound RNA to shifted species (Figure 4.8a). This number is slightly weaker than published values for NS5A binding to model rU\textsubscript{15} and rG\textsubscript{15} oligonucleotides and agrees with values for NS5A binding to the 3’-NTR of HCV.\textsuperscript{57} In contrast, domain II binds NS5A much less tightly, forming just a single microshift, which might be indicative of kinetically labile binding (Figure 4.8.B). Additionally, 79 bp control appears to have essentially no interaction with NS5A, as expected (Figure 4.8.C). These observations support the aforementioned GU content of the given domains. Given these results, it seemed possible that NS5A could attenuate PKR activation by domains III–IV specifically.

Studies have shown that responsiveness in HCV-1b-infected individuals to IFN depends on a particular sequence within NS5A (codons 2209-2248), termed the IFN sensitivity-determining region (ISDR).\textsuperscript{58-60} Cell culture studies have revealed that PKR
**Figure 4.8** Native mobility gel-shift assays of NS5A binding to HCV domains. (A) NS5A binding to full-length IRES and domains III–IV. Trace amounts of radiolabeled RNA were incubated with the indicated concentrations of NS5A and analyzed by 6% native PAGE. (B) NS5A binding to domain II. Assay was conducted as in panel (A). (C) NS5A binding to 79 bp dsRNA. Bottom–strand 79 bp was radiolabeled (p*BS-79 bp) and pre-annealed to excess unlabeled top–strand RNA in all lanes except the first lane. Assay conducted as in panel (A) and analyzed by 8% native PAGE. Anomalous migration of single-stranded p*BS-79 as two bands is not unexpected, as BS-79 bp has the potential for strong self-structure, supported by migration as a single band on a denaturing gel and indirect evidence from *in vivo* experiments.7
activation is inhibited through its interaction with the NS5A IFN sensitivity-determining region (ISDR).\textsuperscript{61-63} This property gives NS5A the potential to inhibit PKR activation independent of RNA binding.

To test for NS5A-mediated inhibition of PKR, we conducted activation assays in the presence of increasing concentrations of NS5A (0-4 µM) and domain II, domains III–IV, or 79 bp control. As shown in Figure 4.9.A and C, profiles for NS5A inhibition of PKR activation were nearly identical for domains III–IV and domain II, with NS5A providing up to 5-fold inhibition for each domain. This suggests that NS5A inhibits PKR activation independent of its ability to bind RNA. Consistent with this notion, NS5A was nearly as effective in inhibiting activation of PKR by 79 bp control (Figure 4.9.B and C).

The mechanism for the RNA-independent mode of NS5A inhibition of PKR activation appears to center on direct interaction of NS5A with the inactive dephosphorylated form of PKR (M.R.S Hargittai and CEC, unpublished results). We note that there is a 20-30% increase in PKR activation at the lowest NS5A concentrations for each of the tested RNAs that precedes the major, inhibitory arm. The origin of this stimulation is unclear.

Lastly, we examined whether NS5A interferes with activated PKR’s ability to phosphorylate its natural substrate, eIF2a. This was tested by varying the order of addition of PKR, dsRNA, NS5a, and eIF2a (Figure B.5). When NS5A is added after autophosphorylation of PKR, but prior to addition of eIF2a (Figure B5, compare lanes 3 and 4 to lanes 5 and 6), no attenuation of eIF2a phosphorylation is observed, indicating that NS5A is unable to prevent downstream events. This supports the ability of NS5A to interact with PKR but not eIF2a.
Figure 4.9 Inhibition of PKR activation by NS5A in the presence of domain II, domains III–IV, or 79 bp. (A) Inhibition of domain II- and domains III–IV-mediated activation of PKR by NS5A. Domain II and domains III–IV concentrations were 2.5 µM. NS5A concentrations were 0, 0.125, 0.25, 0.5, 1, 2 and 4 µM. (B) Inhibition of 79 bp dsRNA-mediated activation of PKR by NS5A. The concentration of 79 bp in all lanes was 0.1 µM, and concentrations of NS5A were 0.06, 0.125, 0.5, 1, 2, and 4 µM. For both panels (A) and (B), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. Phosphorylation activities were normalized to the lane in which NS5A was absent and are provided under the gels. (C) Graphical representation of the phosphorylation activities in panel (A) as a function of NS5A concentration.
4.5 Discussion

4.5.1 Summary of results

HCV IRES RNA has a complex and highly conserved structure that contains long base-paired stretches that could potentially activate PKR, although many of these regions are interrupted by numerous imperfections. There has been some question about the role of HCV IRES RNA as either an activator or an inhibitor of PKR. In this report, we presented evidence that full-length HCV IRES RNA both inhibits PKR activation by a dsRNA mimic, poly I:C, and activates PKR, with activity depending on IRES concentration. We examined individual segments of the HCV IRES for activation of PKR and found that domains III–IV alone activate PKR, consistent with another report, albeit less potently than full-length IRES. Surprisingly, we also found that domain II is a more robust activator than domains III–IV despite the fact that it has fewer base pairs and contains several internal loops and bulges. Mutational analysis and protein mapping techniques were employed to investigate the structural basis of activation by this atypical PKR activator. In an effort to elucidate the importance of PKR activation by domain II, the interaction of NS5A with individual domains of HCV IRES RNA was also studied. We found that, while NS5A binds domains III–IV and full-length IRES much more tightly than domain II, NS5A inhibits PKR equally in the presence of each of these various IRES elements. In Discussion, we present evidence for structural mimicry of A-form dsRNA by domain II of the IRES and a model for how domain II and NS5A may act to regulate host and viral translation during HCV infection.
4.5.2 Domain II has A-form helical properties

It is well-established that in addition to perfect dsRNA, PKR can bind and be activated by RNAs with complex secondary structural features, including internal loops, bulges, and pseudoknots, as well as single-stranded and non-Watson–Crick motifs.\(^7,12\)\(^{14,64}\) It may be the dynamic nature of many RNA structures that enables PKR to accommodate these structural imperfections; for example, A-bulge-induced bends in the middle of RNA helices are straightened upon PKR binding.\(^64\) Additionally, noncontiguous helices within RNA can combine to activate PKR.\(^12,64\) The unifying principle in activation of PKR by these various RNAs may be maintenance of an overall A-form, or dsRNA-like, topology. However, little direct testing of this idea has been conducted.

Domain II of HCV RNA contains four short helical regions, as well as internal loops, bulges, and mismatches, and yet has the ability to activate PKR (Figures 4.1 and 4.3.B). We propose that PKR still binds and is activated by domain II, despite the absence of long base-paired stretches, because the overall conformation of several of these loop regions are largely A-form. We first make a comparison between L4 of domain II and a loop from another RNA known to facilitate binding of a dsRBD, followed by a comparison between the P2–P4 section of domain II and a model A-form helix.

We found p20-dependent protection from nuclease digestion at several nucleotides in the apical loop (L4) of domain II (Figure 4.6.A and C). Mutation of this loop to an unstructured U7 loop resulted in decreased activation of PKR (Figure 4.7). These data prompted a detailed inspection of the NMR structure of domain II\(^{48}\), which
revealed that the apical loop, L4, is fairly structured, with U80, A81, C84, and A85 turned in towards the helix. In addition, U80 and A85 interact via the 2’-hydroxyl of U80 hydrogen bonding with the N6 of A85, and U86 is extruded (Figure 4.10.A). Thus, L4 resembles a tetraloop having sequence AGCC.

There is precedent for other dsRBDs to interact with RNA terminal loops, including those from Staufen, RNase III, Drosha, and ADAR 2. In particular, Feigon and co-workers found that the dsRBD of Rnt1p RNaseIII binds AGNN tetraloops, wherein the conserved A and G do not form sequence-specific contacts with the protein but rather help orient the remainder of the tetraloop to form non-sequence-specific minor-groove contacts primarily via the NN nucleotides. We thus considered the possibility that L4 of domain II, which conforms to the AGNN motif, binds the dsRBD of PKR in a similar fashion.

A structural comparison between the NMR structures of AGCC from L4 and AGAA from Rnt1p-snR47h (small nucleolar precursor substrate of Rnt1p) RNA complex was carried out (Figure 4.10.A and B). We consider first positioning of the bases, followed by positioning of the sugar-phosphate backbone. Overall, the relative positions of the bases in the two tetraloops are quite similar: the A’s at the 5’-end of the loop (A81 from domain II and A15 from snR47h) are oriented inwards and stack over the helix below, the G’s at position 2 of the loop (G82 from domain II and G16 from snR47h) are flipped out of the loop, and the remaining two bases roughly stack over the 3’-end of the helix. In the AGAA tetraloop, the highly conserved G16 is in the syn conformation about the glycosidic bond with the rare C2’-endo sugar pucker. These conformational characteristics do not lead the nucleobase of G16 to make direct contact
Figure 4.10  Structural comparisons of domain II with AGNN loop and 19 bp dsRNA. (A) Secondary structures of domain II L4 and snR47h RNA apical loop employed for RMSD analysis. Numbering for snR47h is from the reference. At left, domain II structure contains a portion of P4 and L4, depicted in its tetraloop-like form, as described in the text, in which U80 hydrogen bonds with A85 (dotted line), and U86 is extruded from the loop. The boxed region contains all the nucleotides assigned to L4 according to the phylogenetically determined secondary structure (see Fig. 1). Loop nucleotides included in the overlay are indicated in red. At right, snR47h RNA AGNN tetraloop, with nucleotides included in the overlay indicated in purple. (B) Overlay of sugar-phosphate
backbones from panel (A). Domain II L4 nucleotides AGCC are in red and snR47h AGAA tetraloop are in purple. Numbering for domain II (PDB ID: 1P5P) is shown adjacent to ribose sugars, with snR47h (PDB ID: 1T4L) numbering in parentheses. All atoms shown were included in RMSD calculations. (C) Secondary structures of domain II P2-P4 region and 19 bp dsRNA employed for RMSD analysis. At left are the regions of domain II included in the comparison. The boxed regions contain all the nucleotides assigned to L2 and L3. Pairing within these loops, as described in the text, is denoted by dashed lines. Nucleotides compared in the overlay are described in Materials and Methods. (D) Stereoview of overlay of sugar-phosphate backbones from panel (C). This structure is sized such that loops and pairing elements approximately align with the secondary structures in panel (C). Lowest-energy NMR structure of domain II is used (PDB ID: 1P5O). Domain II truncations are still in red and 19 bp RNA (PDB ID: 1QC0) is in tan. 2’-Hydroxyls are represented by spheres. Brackets indicate positions of L2 and L3, and the arrow indicates the 2’-hydroxyl of domain II G94. The nucleotides in domain II that were excluded from the alignment (A74, G75, C76, G94, and U106) are shown in lavender (see Materials and Methods). For clarity, nucleotides 20 and 21 of 19 bp RNA are not shown.

with Rnt1p, but they do allow for exposure of the non-bridging phosphate oxygens between G16 and A17. While the corresponding G in L4 (G82) is anti in most of the NMR structures entered into the Protein Data Bank (PDB), the sugar pucker of G82 in the majority of the 12 NMR structures is also C2’-endo resulting in similar exposure of the non-bridging phosphate oxygens between G82 and C83 (Figure 4.10.B).

Next, the sugar-phosphate backbones of AGCC and AGAA were overlaid. We found an RMSD between the two backbones of just 1.57 Å (Figure 4.10.B). Moreover, visual inspection of the overlay reveals good superposition of the 2’-hydroxyls and phosphates. Given that PKR interacts with 2’-hydroxyls and phosphates rather than base moieties, this suggests that the structured nature of the L4 loop may contribute to activation of PKR by domain II through a mechanism similar to that by which AGNN
tetraloops are recognized by Staufen and RNase III.

It should be noted that the effects of the L4–L5 mutation to domain II on PKR activation are subtle, suggesting that PKR binding to the L4 tetraloop may not be critically dependent upon specific recognition of the structural details described above. Indeed, for Rnt1p RNase III, binding affinity for an AGNN versus a nonspecific tetraloop was not drastically different.\textsuperscript{66,69} The stem of the snR47 construct used in the Rnt1p–snR47h NMR structure contains only 15 bp, while in the NMR structure of Staufen dsRBD bound to a RNA tetraloop, the stem is only 14 bp. Given that the dsRBD motif typically requires 16 bp of dsRNA for binding, the minimal nature of the constructs used in these two RNA constructs may in fact ‘force’ the dsRBD of Rnt1p and Staufen to interact with structured loop sequences. This may also be the case for interaction of L4 from HCV IRES domain II with PKR, in which the absence of 33 bp within the stem of domain II required for functional dimerization of PKR necessitates binding to the structured tetraloop. Thus, HCV IRES domain II may be an example of a biological RNA that presents a minimal construct for PKR activation.

Next, we turn our attention to the portion of domain II between P2 and P4. As mentioned, it is possible that activation of PKR by domain II is facilitated by A-form structural mimicry. In fact, the loops in this region of domain II are largely symmetrical—L2 is a 2x2 loop and L3 is a 3x4 loop—opening the possibility for nearly bulge-free non-Watson–Crick base pairing. Indeed, the NMR structure of domain II reveals a stretch of noncanonical base pairs within L2 and L3.\textsuperscript{48} In particular, L2 consists of two pyrimidine–pyrimidine base pairs, U63·C104 and U64·U103, while L3 contains three non-Watson–Crick base pairs, A73·A93 (AA N7 symmetric), A72·U95 (AU
Hoogsteen), and G71:A96 (sheared GA). In fact, the only base in L2 or L3 that does not directly participate in base pairing is G94, which was the only base within L3 that was not protected from p20-dependent in-line cleavage (Figure 4.6.C). These observations suggest that, despite their noncanonical nature, PKR may recognize the base pairs in L2 and L3 as dsRNA. We do note, however, that the identity of the bases in these loops seems to be relatively unimportant, as substitutions in L2 and L3, which maintained their symmetrical geometries, led to only minor effects on activation.

To further explore A-form mimicry within L2 and L3, we overlaid the P2–P4 portion of domain II with a crystal structure of a model 19-bp A-form dsRNA\(^70\) (Figure 4.10.C and D). The backbones of these two structures gave an overall rmsd of 3.03 Å. In particular, the lower portion of these structures, which contains L2, overlays very well. Visual inspection of this region (Figure 4.10.D) reveals very similar positioning of the 2’-hydroxyls within L2 and model dsRNA, the only significant difference being the width of the two helices, which is ~ 11 Å between 2’-hydroxyls for the A-form helix and ~9 Å for L2, induced by the smaller pyrimidine–pyrimidine base pairs.\(^71\)

The deviation between domain II and model dsRNA is greater near L3, likely due to the curvature of domain II induced by L3. Visual inspection of this overlay region, however, still suggests that the 2’-hydroxyl group positions within L3 are highly similar to those of an overall A-form geometry, with only a few exceptions (Figure 4.10.D, lavender residues). One exception in particular, G94 (Figure 4.10.D, arrow), is not protected by p20, as described above. In summary, domain II from P2 through P4, appears to mimic A-form dsRNA and contributes a potential 22 A-form-like base pairs.

Below P2 are L1 and P1. In contrast to L2 and L3, L1 is a 5-nucleotide bulge,
which leads to a kink in the backbone, although this region is likely flexible as is characteristic of large bulges. The role of L1 in activation is unclear. Protections mapped onto the NMR structure in Figure 6 suggest that the dsRBMs of a PKR dimer interact extensively with domain II, with the exception of L1; also, L1 can be deleted with just a slight loss in activation. Thus L1 likely plays little direct role in activation, although it remains somewhat surprising that it can be tolerated in this activating element.

At the base of domain II is the P1 pairing, which contributes 8 Watson–Crick base pairs. Together with the 22 A-form-like base pairs from P2 through P4, this gives 30 bp total. Assuming that L4 interacts productively with p20, as proposed above, an effective total number of base pairs is ~33, which is the minimum number needed for activation of PKR. Similarly, addition of base-paired segments leading to this value were found for the IFN-g mRNA pseudoknot. Thus, the ability of shorter base-paired segments and symmetrical loops to sum to a PKR activating total appears to be a common theme.

Lastly, we briefly consider the weaker activation of PKR by domain III–IV of HCV IRES. This domain contains more extensive helical regions than domain II (Figure 4.1), yet it is a less potent activator of PKR (Figure 4.3). We suggest that this may be due to the branched nature of this domain, which contrasts with the unbranched domain II. In particular, structural motifs within domain III of the IRES appear to resemble motifs in well-studied PKR-inhibitory RNA structures. Specifically, the geometries of the four-way junction from IIIabc and the three-way junction composed of the branched IIId stem–loop from HCV IRES (Figure 4.1) parallel portions of the inhibitory Epstein–Barr EBER1 RNA and adenovirus VA1 (noncoding virus-associated RNA I from adenovirus) RNA. EBER1 contains a four-way junction composed of two stem regions and two
branched stem-loops, with one shorter than the other, while the central domain of VA1 RNA contains a three-way junction that is critical for PKR inhibition. Thus, the two multi-junction regions in domain III may cause PKR to adopt a combination of activating and inhibiting binding geometries, leading to its poorer activation.

4.5.3 Possible roles for PKR in HCV replication

It is known that NS5A regulates PKR activation throughout the lifecycle of HCV. Observation that NS5A can equally inhibit activation of PKR by domains II and III–IV suggests possible models for involvement of these IRES domains in PKR activation. In Table 1, we present possible modes for regulation of host and virus translation through PKR and NS5A interaction. Early in the viral life cycle, when NS5A levels are low, PKR could be activated by the IRES leading to phosphorylation of eIF2a and inhibition of cap-dependent host translation. However, viral translation, which has been shown to function independent of PKR phosphorylation, would continue unabated, leading to high levels of viral proteins (Table 4.1, column 2). Later in the viral life cycle, when NS5A levels have increased, PKR activity would be inhibited, leading to upregulation of host translation but still allowing high levels of viral translation. In this way, early in infection translation would be predominantly of viral proteins, but host proteins would be made available to the virus for later stages of replication.

We demonstrated that NS5A binds tightly to domains III–IV but only weakly to domain II. One possible consequence of this is that low levels of NS5A could be sequestered by binding to domains III–IV, allowing PKR activity to remain high via activation by domain II. In fact, the spacing of the bands in Figure 4.8 suggests that
Table 4.1 Mediation of host and viral translation through NS5A-PKR interactions

<table>
<thead>
<tr>
<th>Viral life cycle:</th>
<th>Early</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS5A levels:</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>PKR activity:</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Host translation:</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Virus translation:</td>
<td>high</td>
<td>high</td>
</tr>
</tbody>
</table>

domains III–IV can titrate 3 or more NS5A proteins, consistent with the presence of 5 GU-rich elements in this region. Only later in infection, when NS5A levels become high enough to exceed the concentration of binding sites, would PKR be bound by NS5A and be inhibited. Weaker binding of NS5A to PKR than RNA is suggested by the 50% values for RNA binding and PKR inhibition (Figures 4.8.A and 4.9.C). This model for the interaction of PKR, NS5A, and IRES elements is consistent with observation that domain II is required for both replication and translation, whereas domains III–IV are only required for translation. Indeed, it has previously been suggested that domain II could function as a switch between translation and replication. Given the evidence here presented, it is possible that this domain II regulation of HCV translation and replication is mediated by differential interaction with PKR and NS5A.

Contributions: RT conducted all writing and experiments in text and Appendix B with these exceptions: SRN performed inhibition and activation assays on full-length IRES and in the presence of NS5A and 5’-triphosphate-dependence assay in text and Appendix B, and contributed to writing. JAB performed alignments and obtained RMSD values.
4.6 Acknowledgments

We thank Daniel G. Cordek and Suresh D. Sharma for providing purified NS5A proteins. This study is supported by National Institutes of Health Grant R01-58701.

4.7 References


Appendix B

Supporting Information: Chapter 4

[Published as Supporting information for a paper entitled “Regulation of PKR by HCV IRES RNA: Importance of Domain II and NS5A” by Rebecca Toroney, Subba Rao Nallagatla, Joshua A. Boyer, Craig E. Cameron, and Philip C. Bevilacqua in Journal of Molecular Biology 2010 400: 393-412.]

**Figure B.1** Activation of PKR by domain II is not 5’-triphosphate-dependent. Activation assay using domain II transcript. RNAs were transcribed and untreated (5’-ppp) or CIP-treated (5’-OH). A no-RNA lane (−) is provided. Phosphorylation activities are normalized to 0.1 mM 79 bp. 10% SDS-PAGE gel is shown.
Figure B.2  Native mobility gel-shift assays of p20 binding to HCV domains. (A) PKR dsRBD (p20) binding to full IRES. (B) p20 binding to domains III-IV. (C) p20 binding to domain II. For all panels, trace amounts of radiolabeled RNA were incubated with the indicated concentrations of p20 and analyzed by 6% or 20% native PAGE, as indicated.
Figure B.3 Graphical representations domain II of footprinting data presented in Figure 4.6.A and B. (A) Graphical representation of RNase A and V1 protections. For each RNase, – and + lanes from Figure 4.6.A were quantified, normalized for loading, and then plotted. Beneath the plots, pairing and loop regions that correspond to secondary structure elements in Figure 4.6.C are indicated. (B) Graphical representation of in-line probing protections. For 0.625 µM (or 10 µM) p20, lanes from Figure 4.6.B were quantified, normalized for loading, and then plotted. Beneath the plots, numbering of domain II primary sequence is indicated.
Figure B.4 Activation of PKR by domain II mutants in L1, L2, and L3. (A) Mutants (C104U, G71A/G94A, and ∆L1) superimposed on secondary structure of domain II wild-type (WT). (B) PKR activation assays of L1, L2, and L3 mutants (∆L1, C104U, G71A/G94A) mutants. RNA concentrations for WT and mutants were 0.31, 0.6, 1.5, 2.5, 5, and 10 µM. Phosphorylation activities for both gels were normalized to 0.01 µM 79 bp in lower gel and are noted under the gel. 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. (C) Graphical representation of phosphorylation activities from panel (B) as a function of RNA concentration.
**Figure B.5** Activation of PKR and phosphorylation of eIF2α by 79 bp dsRNA in the presence of NS5A. Order of addition for each lane is described according to the schematic: (Lane 1) 79 bp dsRNA (0.1 µM, final), λpp-treated PKR (0.8 µM, final), and ATP were incubated for 10 min, followed by addition of protein storage buffer (PSB) and incubation for 10 min. Finally, eIF2α (8 µM, final) was added and the reaction was quenched after 3 min of incubation. (Lane 2) Same as in lane 1, except 5 min incubation. (Lane 3) 4 µM NS5A and λpp-treated PKR (0.8 µM, final) were incubated for 10 min, followed by addition of 79 bp RNA (0.1 µM, final) and ATP and incubation for 10 min. Finally, eIF2α (8 µM, final) was added and the reaction was quenched after 3 min. (Lane 4) Same as in lane 3, except 5 min incubation. (Lane 5) 0.1 µM 79 bp RNA (0.1 µM, final), λpp-treated PKR (0.8 µM, final) and ATP were incubated for 10 min, followed by addition of NS5A (4 µM, final) and incubation for 10 min. Finally, eIF2α (8 µM, final) was added and the reaction quenched after 3 min. (Lane 6) Same as in lane 5, except 5 min incubation. Denaturing 10% SDS-PAGE gel is shown and positions of phosphorylated PKR (p-PKR) and eIF2α (p-eIF2α) are indicated.
Chapter 5

Effects of additional RNA structures on PKR function: (1) HCV IRES tertiary structure and (II) Non-Watson-Crick mismatches

5.A Abstract

The preceding chapters have addressed the role of various RNA primary and secondary sequence elements in PKR activation. The first part of this chapter expands on Chapter 4, which focused on the role of multiple secondary structure defects within domain II of HCV IRES. Here the effects of two tertiary elements located in domains III-IV of the IRES on PKR activation by full-length HCV IRES RNA are investigated. In the second part of this chapter, the potential role in PKR regulation by one specific secondary structure defect, the tandem GA mismatch, is explored. Tandem mismatches are essentially symmetric [2x2] internal loops, and thus this portion of the chapter also explores an idea from Chapter 4, that PKR is tolerant of symmetric defects in RNA.

5.1 Determining the role of an extended RNA tertiary structure on PKR activation by hepatitis C virus internal ribosome entry site (HCV IRES) RNA

5.1.1 Abstract

The RNA-activated protein kinase, PKR, functions as an integral member of the innate immune response in humans through recognition of non-self RNA, which it senses in a non-sequence-specific fashion via its double-stranded RNA binding domain (dsRBD). Activation of PKR is characterized by binding of two PKR monomers on a single RNA molecule, which promotes dimerization and activation of the kinase with the downstream effect of translation initiation inhibition. Despite the designation “double-
stranded RNA binding domain”, RNAs containing a wide variety of secondary and even tertiary structural features have been identified as binders and regulators of PKR. One such activator is the internal ribosome entry site of hepatitis C virus (HCV IRES), an RNA that consists of multiple, independently folded secondary domains. Although various of these individual domains have been identified as activating species within the IRES, the structural nature of PKR’s interaction with the full-length IRES remains ambiguous, particularly in light of the tertiary structural elements known to exist within this RNA. In the present study, we utilized two point mutations known to disrupt the tertiary fold of the IRES to examine the effects of tertiary structure on HCV IRES-dependent PKR activation. The Mg\(^{2+}\)-dependent PKR activation and UV melting profiles of these mutant RNAs were determined and suggest that tertiary structural elements of the IRES may somewhat promote PKR activation, although HCV IRES secondary structure alone is still sufficient for activation. The modest contribution of the IRES tertiary fold to PKR activation is consistent with its overall extended, as opposed to globular, nature and thus with its resultant resemblance to a more prototypical dsRNA activator.

5.1.2 Introduction

Interactions between proteins and RNA are critical for many fundamental cellular processes. The hierarchical nature of RNA folding, whereby primary sequence folds into secondary structure followed by tertiary structure, adds a layer of complexity to the myriad of RNA-protein binding modes.\(^1\) A vast array of RNA-protein complexes have
been identified in which RNA primary, secondary, or tertiary structure, or some combination of the three levels, serve as determinants for protein recognition and binding. Some of the more ubiquitous protein RNA-binding motifs include the RRM, or RNA-recognition motif, which binds primarily to single-stranded RNA, and the dsRNA binding motif, or dsRBM, which binds non-sequence-specifically primarily to duplex RNA, including complex RNA secondary structures containing imperfections such as bulges and internal loops.

The protein kinase R (PKR) is an RNA-binding protein kinase containing two tandem dsRBMs that is part of the innate immune response in humans and functions principally in recognition of viral RNA of at least 33 bp in length. PKR is activated through binding of RNA by the dsRBMs of two PKR molecules, which leads to kinase dimerization and autophosphorylation. Activated PKR then phosphorylates its protein substrate, eIF2α, which results in the shut-down of cellular translation. Besides strictly duplex RNA, PKR has been shown to recognize a variety of features at various levels of the RNA structural hierarchy. For example, largely single-stranded RNAs containing short stem-loops have been found to activate PKR in a 5′-triphosphate-specific manner, while certain internal nucleoside modifications have been shown to modulate PKR activation by both single- and double-stranded RNA. Additionally, PKR is regulated by viral RNAs with complex secondary structures, including adenovirus VA1 RNA and hepatitis C virus internal ribosome entry site (HCV IRES) RNA. Both RNAs contain bulges, internal loops, and multi-helix junctions, as well as tertiary structure, but whereas VA1 RNA is a potent inhibitor by binding PKR monomers into complexes incapable of promoting kinase dimerization, HCV IRES RNA is a potent activator of PKR.
The secondary and portions of the secondary structure of the HCV IRES (nt 1-388) have been defined phylogenetically, biochemically, and structurally (Figure 5.1.1), and PKR activation by HCV IRES RNA has been characterized through domain studies in our laboratory which it was determined that while domains III-IV is capable activating PKR, domain II alone is the more potent activator. Neither of these domains, however, activates PKR as potently as the full-length IRES, which suggested that some further level of structural complexity contributes to PKR activation by full-length HCV IRES. Interaction of PKR across multiple IRES domains or with the IRES tertiary structure are two possibilities.

While no structure of the full-length HCV IRES has yet been solved, chemical and enzymatic probing have provided evidence for a tertiary fold that forms at physiological Mg\(^{2+}\) concentrations. Additionally, small angle x-ray scattering (SAXS) supports the presence of an IRES tertiary fold that is extended and flexible, as opposed to a more typical globular fold. A number of point mutants known to interfere with the ability of HCV IRES to function in viral translation initiation were subjected to ion-induced FE(II)-EDTA and nuclease protections assays, and two mutations in particular, U228C and G266C, were found to interfere with proper folding of the IRES tertiary structure.

A crystal structure of the IIIabc four-way junction in domain III of the IRES, in which U228 is located, has been solved and revealed a number of tertiary interactions between junction nucleotides (Figure 5.1.1, top). In particular, U228 forms a noncanonical base pair with A155 across the helix junction, while additional tertiary
Figure 5.1.1 Crystal and NMR structures of HCV IRES tertiary domains in secondary structure context. **Left.** Outline of HCV IRES 1-388 secondary structure, with domains numbered in Roman numerals and location of junction IIIabc and domain IIId boxed. **Top.** Left: Sequence and secondary structure of IIIabc and flanking nucleotides, with nucleotides included in crystal structure in magenta. U228 is indicated in blue and is involved in a non-Watson-Crick base pair (dashed gray line) with A155. Other nucleotides involved in tertiary contacts (A154, A155, A172, A173) are in yellow. Right: Crystal structure of junction IIIabc from two different views (front view and helical axis view), with nucleotides colored according to secondary structure. **Bottom.** Left: sequence and secondary structure of domain IIId and flanking nucleotides, with nucleotides included in NMR structure in magenta, and G266, which is implicated in tertiary contacts between IIId and other IRES elements, in green. Right: NMR structure of domain IIId with G266. U228C and G266C mutations, which disrupt these tertiary domains, are indicated with arrows on respective secondary structures.
contacts are formed between A155, A154, A172, and A173. These interactions serve to produce an overall “X” shape created by two sets of helical stacks. The site of the other tertiary mutation, G266, is located within domain IIId, the structure of which has been solved by NMR (Figure 5.1.1, bottom). Although possible tertiary contacts form with nucleotides in domain IIId within the context of the full IRES, as evidenced through the aforementioned protection assays, they are in as yet undetermined regions of the IRES and are therefore not depicted in this structure.

Given the presence of these tertiary elements and the superior activation of PKR by full-length HCV IRES RNA versus individual secondary domains, we sought to determine effects of IRES tertiary structure on regulation of PKR by utilizing the two point mutations known to disrupt IRES tertiary elements, U228C and G266C. We also investigated effects of Mg²⁺ concentration on PKR activation by these mutants and on UV melting profiles of the mutant IRES RNAs. The data suggest that the IRES tertiary structure plays a subtle role in activating PKR, which we attribute the extended, as opposed to globular, nature of the HCV IRES tertiary structure, which renders it more similar in overall shape to A-form helical RNA.

5.1.3 Materials and Methods

5.1.3.1 RNA Sequences

The 79 bp (dsRNA-79) control was prepared by transcribing opposing strands of pUC19 and annealing, as previously described. The remaining RNAs were prepared by
*in vitro* transcription from a linearized plasmid (see below). Following are the sequences of RNA used in this study.

**HCV IRES 1-388**

Wild-type sequence. Underlined boldfaced nucleotides denote positions of tertiary mutations, U228C and G266C, respectively. Double mutant contained both mutations in the same RNA.

```
5’GCCAGCCCCCGAUUGGGGGCGACACUCCACAUAGAUCACUCCCCUGUGAGA
GGAACUACUGUCUUCACGCAGAAAGCGUCUAGGCGUUAUGAG
UGUCGUUGCAGCCUCAGGACCCCCCCUCCCCAGGAGAGCAGGCAUGUGCUCGC
GGAACCGGGAGUACCCCGAAUUUGCCAGACGAGCCGGGUGCCGUUUUCUUGG
AUCACCCCGUCUAAUGCCUGAGAUUUGGGCUGCCGGCCGACUAGCUA
GCCGAGUAGUGUGUGGCUGCGAAAGGCUCUUGUGUAGCCGCAUAGGGG
GCUUGCGAGUGCCCGGAGGUCUCGUAGCAGCCGGCAGCAGCGAAG
UCCUAAACCUCAAAGAAAAACCAAAGGGCGCGCC
```

**5.1.3.2 RNA preparation**

Wild-type HCV IRES (1-388) was transcribed by run-off transcription using linearized pUC18 plasmid (*Bam*H1 digested) containing a T7 promoter. Mutant HCV 1-388 RNAs (U228C, G266C, and U228C/G266C) were similarly transcribed from mutant plasmids generated using the QuikChange site-directed mutagenesis kit (Stratagene). Sequences of mutant and wild-type plasmids were confirmed by dideoxy sequencing following maxipreps and minipreps. For each RNA, 1.5 µg of linearized plasmid was incubated with 1.8 µg of T7 RNA polymerase, 40 mM Tris (pH 8), 33 mM Mg(OAc)$_2$, 40 mM DTT, 2 mM spermidine, and 7 mM of each NTP at 37 °C. After 3.5-4 h, the reaction was quenched by adding an equal volume of 95% (v/v) formamide loading buffer. RNA was then purified by fractionating on a polyacrylamide denaturing gel (7 M urea, 1X TBE). The transcript was identified by UV shadowing, excised from the gel, and eluted overnight at 4 °C in 1X TEN$_{250}$. The RNA was then ethanol
precipitated and resuspended in 1X TE buffer (pH 7.5) and stored at –20 °C. RNA concentration was determined spectrophotometrically.

5.1.3.3 Protein preparation

Full-length PKR and the dsRBD (p20) containing an N-terminal His\textsubscript{6} tag were purified from \textit{Escherichia coli} BL21(DE3) Rosetta cells (Novagen) as described previously.\textsuperscript{5,19} The concentration of proteins was determined spectrophotometrically.\textsuperscript{20}

5.1.3.4 PKR activation assays

RNAs were tested for their ability to activate PKR autophosphorylation. PKR was first dephosphorylated by treatment with $\lambda$-PPase (NEB) for 1 h at 30°C, followed by addition of freshly made phosphatase inhibitor, sodium orthovanadate.\textsuperscript{21} Next, 15 $\mu$Ci $[\gamma$-$^{32}$P]ATP (Perkin-Elmer), 0.6 $\mu$M dephosphorylated PKR and RNA were incubated in 20 mM HEPES (pH 7.5), 4 mM MgCl\textsubscript{2}, 100 mM KCl, and 100 $\mu$M ATP (Ambion) for 10 min at 30 °C. In certain cases, the final concentration of MgCl\textsubscript{2} was varied from 0.5 to 4.2 mM, all in the background of physiological concentrations of KCl (100 mM) to promote secondary structure formation. All reactions were quenched by addition of SDS loading buffer. Samples were heated at 95°C for 5 min then loaded on 10% SDS-PAGE gel (Pierce). After electrophoresis, gels were exposed to a storage PhosphorImager screen and intensities of labeled bands were quantified on a PhosphorImager (Molecular Dynamics). A background value was averaged from different portions of the gel and subtracted from each band prior to normalization.
5.1.3.5 UV absorbance-detected thermal melting of RNAs

Melting profiles were obtained at 260 nm on a Gilford Response II spectrophotometer using a 0.5 cm cuvette and 0.25 µM RNA, with a data point acquired every 0.5 °C. RNA was renatured prior to melting by heating at 90 °C for 3 min followed by slow cooling to room temperature for 10 min. RNA was then added to the following buffer components and heated at 55 °C for 2 min, then slow cooled to room temperature: 10 mM Tris buffer (pH 7.0), 0.1 mM EDTA, 100 mM KCl, and 0.5 to 4.2 mM MgCl₂. Data were plotted on Kaleidagraph, and after 7-point smoothing, the first derivative was then plotted.

5.1.4 Results and Discussion

5.1.4.1 Mutations that disrupt RNA tertiary structure mitigate PKR activation by HCV IRES RNA

Previous studies have established that HCV IRES RNA functions both as an activator and an inhibitor or PKR, with the effect dependent upon IRES concentration: IRES activates maximally near ~0.6 µM, and inhibits at higher concentrations (Figure 5.1.2, purple trace; this trace is from published data¹³). This bell-shaped dependence of PKR activation on RNA concentration is typical of perfect dsRNA activators and can be attributed most simply to titration of PKR dimers onto individual RNA molecules as monomers, rendering PKR inactive. It was reported previously that the folding transition for HCV IRES tertiary structure occurs between 0.5 and 1 mM Mg²⁺, and that at 2.5 mM Mg²⁺ HCV IRES runs as a single band on a native gel.¹⁵ In our standard activation
assays, the concentration of Mg$^{2+}$ is 4.2 mM, suggesting that the IRES tertiary structure should be fully folded, without stable alternative structures or higher order aggregates.

In order to probe the effect of HCV IRES tertiary structure on PKR activation, we transcribed HCV IRES 1-388 with a U228C or a G266C mutation, which have been reported to disrupt proper tertiary folding, and tested these RNAs for PKR activation. We observed that while both mutant RNAs are still capable activators of PKR, the bell-shaped concentration dependence of activation was shifted to 4-fold (0.63 to 2.5 µM maxima) higher RNA concentrations relative to the wild-type (WT) IRES (Figure 5.1.2.A and B). The requirement of higher RNA concentrations for maximal activation implies weaker affinity of PKR for these mutants, suggesting a positive contribution of HCV IRES tertiary structure to PKR activation by WT IRES. This observation suggests that tertiary structure of the IRES promotes PKR activation.

5.1.4.2 Mg$^{2+}$-dependence of PKR activation by WT IRES and tertiary mutants

Proper folding of RNA tertiary structure is often dependent upon the concentration of divalent metal ions. It has been shown that Fe(II)-EDTA and RNase T1 cleavage protection patterns of HCV IRES change in the presence of Mg$^{2+}$, and that these protection patterns differ from the Mg$^{2+}$-dependent protection patterns of U228C and G266C mutants, providing evidence that these two point mutations alter IRES tertiary structure. In particular, Keift et al. reported Mg$^{2+}$ K$_d$ of 0.7-0.8 mM (Hill coefficient ~2.2-2.9) by hydroxyl radical probing, and a Mg$^{2+}$ requirement of 0.25 mM by RNase protection T1 mapping. We were interested to see if a similar disparity exists
**Figure 5.1.2** Effect of HCV IRES tertiary mutants on PKR activation. (A) PKR activation assays by HCV IRES 1-388 U228C (upper gel, right hand lanes) and 1-388 G266C (lower gel). RNA concentrations for U228C were 0.16, 0.32, 0.63, 1.3, 2.5, and 5.0 µM, and concentrations for G266C were 0.16, 0.32, 0.63, 1.3, 2.5, and 4.0 µM. 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. No-RNA, 79 bp dsRNA, and 1-388 wild-type lanes are included at the indicated RNA concentrations. Phosphorylation activities are normalized to the 79 bp lane and noted under the gels. (B) Graphical representation of phosphorylation activities from panel (A) and activation assay of 1-388 wild-type (raw data from reference 13 and Chapter 4) as a function of RNA concentration. Assay conducted in 100 mM KCl and 4.2 mM Mg²⁺.

among the Mg²⁺-dependence of PKR activation by wild-type and mutant IRES RNAs, and so performed activation assays across a range of MgCl₂ concentrations for each RNA, as well as a 79 bp dsRNA standard (**Figure 5.1.3**). We overall observe an increase in PKR activation with increasing Mg²⁺ concentration across all RNAs, which is to be expected given that PKR generally requires mM quantities of divalent ion for activation (**Figure 5.1.3.A and B**).
In order to compare more directly effects of Mg\(^{2+}\) due to RNA folding, rather than PKR magnesium requirement, we divided the percent activation at each Mg\(^{2+}\) concentration for by the corresponding percent activation for WT IRES to give the “fold-down” PKR activation over WT (Figure 5.1.3.C). First, the activation for 79 bp is largely not dependent on Mg\(^{2+}\). This suggests that between 0.5 and 4.2 mM Mg\(^{2+}\) the WT HCV IRES is largely folded as expected from earlier studies on RNase T1 probing.\(^{15}\) In contrast, fold-down activation by the two mutants increases with decreasing Mg\(^{2+}\) concentration, suggesting that loss of tertiary structure in IIIabc and IIId is less compatible with activation. Indeed, increasing Mg\(^{2+}\) concentration to 4 mM compensates for the loss of activation (Figure 5.1.3.C, green and blue symbols level off). This effect is likely due to the restoration of tertiary structure of the mutants relative to WT, particularly for the U228C mutation, where 2.5 mM Mg\(^{2+}\) has been shown to restore RNA tertiary WT folding.\(^{15}\) (It should be noted that higher activation by G266C is actually higher than WT at 2.5 mM Mg\(^{2+}\) does not imply that G266C is overall a better activator than WT, because the RNA concentration for G266C is 2-fold higher than WT in this assay.)

The Mg\(^{2+}\)-dependent activation profiles of U228C and G266C suggest that there may still be tertiary structure present in each of these mutants that contributes to activation of PKR by the IRES. This notion is consistent with isolated locations within the IRES of the proposed tertiary features of each of these mutations: U228 participates in cross-junction base interactions that result in tight coaxial helical stacking, while G266 is likely involved in separate tertiary interactions not directly associated with the IIIabc junction.
Figure 5.1.3 Mg²⁺-dependence of PKR activation by HCV IRES 1-388 wild-type and tertiary mutants. (A) PKR activation assays by 1-388 wild-type (WT), 1-388 G266C (G266C), and 1-388 U228C (U228C) at 0.5, 1, 2, and 4.2 mM Mg²⁺. No-RNA and 79 bp dsRNA were also assayed at the same Mg²⁺ concentrations. Separate no-RNA and 79 bp dsRNA lanes are included as controls (last two sets of lanes). RNA concentrations were as follows: 1.25 µM WT, 2.5 µM G266C and U228C, and 0.1 µM 79 bp, as indicated on gels. The 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. Phosphorylation activities are normalized to the 79 bp dsRNA control lane and noted under the gels. (B) Graphical representation of phosphorylation activities from panel (A) as a function of Mg²⁺ concentration. (C) Fold-effect of Mg²⁺ on PKR activation versus 1-388 wild-type. For 1-388 U228C and G266C, the percent activation at each Mg²⁺ concentration was divided by the corresponding percent activation for 1-388 wild-type to show the change in “fold-down” PKR activation by tertiary mutants as a function of Mg²⁺ concentration. The same procedure was conducted for 79 bp dsRNA as a control.
Indeed, structure probing supports separation of the elements. Thus, disruption of tertiary elements by one of these mutants allows formation of tertiary interactions elsewhere in the IRES, i.e. at the site of the other mutation while the other tertiary element is rescuable by Mg\(^{2+}\).

### 5.1.4.3 UV-dependent thermal denaturation of HCV IRES WT and mutants

In order to probe the notion that the IRES tertiary mutants may still have elements of tertiary structure that contribute to PKR activation, we performed thermal denaturation experiments for WT, U228C, and G266C IRES RNAs at four different concentrations of MgCl\(_2\). Melts and their first derivatives are provided in Figure 5.1.4. For WT IRES, the major unfolding transition occurs between 60-80 °C, which likely represents unfolding of the extensive IRES secondary structure and the T\(_m\) increases from ~62 to 70 °C with Mg\(^{2+}\) concentration from 0.5 to 4 mM (Figure 5.1.4.A and B).

Below 40 °C there appears to be a series of small transitions that change with Mg\(^{2+}\) concentration, that could represent melting of IRES tertiary structure, different elements of which may have different thermal stabilities. These transitions, however, are also present for the two tertiary structure-disrupting single mutants U228C (Figure 5.1.4.C and D) and G266C (Figure 5.1.3.B), suggesting these transitions may just be noise and that tertiary structure melting instead overlaps with the secondary structure transition.

Overall the melting profiles for the WT and mutant IRES constructs are very similar, suggesting that effects of mutants on the overall structure of the IRES are subtle,
**Figure 5.1.4** UV absorbance thermal denaturation and derivative curves of HCV 1-388 WT, U228C, and G266C with varying Mg$^{2+}$. Panels A, C, and E show the UV melting curves for WT, U228C, and G266C, respectively, while panels B, D, and F show the associated derivative plots for A, C, and E, respectively. Melts were conducted in Tris buffer (pH 7.0), 100 mM KCl, and 0.1 mM EDTA.
consistent with the extended nature of IRES tertiary structure. It is noteworthy that despite the apparent subtle effect of these tertiary mutants on the overall structure of 5.1.4.4 Effect of double mutation on PKR activation by HCV IRES

Melting profiles and previously reported structure mapping\textsuperscript{15} suggests that there are likely still elements of tertiary structure present in each of these U228C and G266C. We thus prepared the double mutant U228C/G266C, and tested for PKR activation (Figure 5.1.4). The double mutant displayed similar RNA concentration requirements as the two single mutants (Figure 5.1.5) with even slightly greater PKR activation at higher and lower RNA concentrations. Thus inhibitory effects of the two tertiary structure mutants are not additive, although the activation profile is broader for the double mutant (Figure 5.1.4.B).

A possible explanation for these observations is that while the tertiary elements of HCV IRES contribute to PKR recognition and activation, disruption of individual elements of the tertiary structure via the single mutants results in an overall IRES shape that is less extended overall, which slightly attenuates activation. Further disruption of the tertiary structure via the double mutant perhaps increases the flexibility of the IRES such that more binding modes are available to PKR, thus slightly restoring PKR activation relative to the single mutants and leading to a widened activation profile.
Figure 5.1.5  Effect of tertiary double mutant (DM) on PKR activation. (A) PKR activation assay by HCV IRES 1-388 U228C/G266C. RNA concentrations were 0.32, 0.63, 1.3, 2.5, 5, and 7.2 µM. 10% SDS-PAGE gel is shown, with position of phosphorylated PKR (p-PKR) indicated. A no-RNA lane and a 79 bp dsRNA lane are included. Phosphorylation activities are normalized to the 79 bp lane and noted under the gel. (B) Graphical representation of phosphorylation activities from panel (A) as a function of RNA concentration. Also included for comparison are phosphorylation activities for HCV IRES 1-388 WT, U228C, and G266C from Figure 5.1.2.
5.1.5 Conclusions

Regulation of PKR by HCV IRES RNA has been a somewhat controversial subject in the literature over recent years. Some reports identified the IRES as a viral inhibitor of PKR activation, similar to VA₁ and EBER RNAs produced by adenovirus and Epstein-Barr virus, which are PKR inhibitors. More recently, HCV IRES has been identified as an activator of PKR. While one report identified domains III-IV of the IRES as the major activating species, we assigned that function primarily to domain II (see Chapter 4). Clearly, the complexity of RNA structure contributes to difficulties in characterizing the regulatory role of HCV IRES RNA in innate immunity. In addition to its extensive and diverse secondary structure, a further layer of complexity arises from its tertiary structure. Although PKR primarily binds to stretches of perfect dsRNA, there is precedent for tertiary structural elements functioning in activation and inhibition. Activation by interferon-gamma (IFN-γ) mRNA requires the formation of a pseudoknot, while inhibition by VA₁ RNA requires a tertiary structure.

We sought to determine the role of tertiary structure in the regulation of PKR by HCV IRES RNA. Our data suggest that two elements of the IRES tertiary structure enhance PKR activation, possibly by stabilizing the IRES in an elongated structure that more closely resembles long dsRNA. In particular, formation of tertiary structure at junction IIIabc results in stacking of the shorter helices around the junction, which is similar to coaxial stacking of shorter helices reported as contributing to PKR activation by other RNAs, including IFN-γ. Disruption of multiple IRES tertiary elements, however, had no additive effect and even slightly restored the level of activation relative to disruption of a single tertiary element. In this case, PKR can still be activated by
individual elements of the IRES secondary structure.

It appears likely that HCV IRES RNA activates PKR through multiple modes, with activation optimized for full-length IRES with tertiary structure fully intact. The ability of PKR to be activated by multiple structural elements of this highly complex RNA may be beneficial to the cell, as it adds an adaptive ability to innate immunity. As accessibility of the IRES to PKR changes over the course of the viral life cycle, the existence of multiple modes of activation potentially allows PKR activation to proceed at multiple stages: cotranscriptionally before the IRES is fully formed, or when portions of the IRES are sequestered by binding of viral proteins or the ribosome.
5.2 Exploring the effect of tandem GA mismatches on PKR activation

5.2.1 Abstract

As an essential part of the innate immune response in humans, the dsRNA-activated protein kinase PKR is known to function as a viral sensor in which it recognizes various RNA PAMPs, or pathogen associated molecular patterns, which promote PKR dimerization and autophosphorylation with the downstream effect of translation inhibition. A number of these RNA molecular patterns have been identified, including long stretches of dsRNA common to viral RNAs,\textsuperscript{7} as well as the 5’-triphosphate of certain mostly single-stranded RNA.\textsuperscript{8} Conversely, certain internal nucleoside modifications and cellular 5’-end modifications lead to abrogation of PKR activation and thus can serve to discriminate cellular and pathogenic RNA.\textsuperscript{9} Another possible means by which PKR can differentiate self and non-self RNA is through non-Watson-Crick motifs.

It has been demonstrated that a biologically rare tandem AG mismatch (\textsuperscript{5}AG\textsuperscript{3})\textsubscript{5} embedded within dsRNA can support potent PKR activation,\textsuperscript{27} whereas multiple GU mismatches, which are phylogenetically common, abrogate the PKR response.\textsuperscript{9} Analogous to the differential prevalence of these two non-Watson-Crick motifs is the tandem GA mismatch (\textsuperscript{5}GA\textsuperscript{3})\textsubscript{3} which, depending on the identity of the flanking base pairs, can adopt one of two distinct geometries, the sheared geometry which is common in biological RNAs, and the imino paired which is absent.\textsuperscript{28-30} Herein we investigate the role of these two types of tandem GA mismatch in the regulation of PKR. Effect of single and multiple GA mismatches of both geometries embedded within several dsRNA constructs are tested. Although it was hypothesized that a GA mismatch with the
underrepresented imino geometry would preferentially regulate PKR, regulation by the imino geometry was only slightly favored over sheared. The geometries of RNA motifs that preferentially regulate thus appear to not simply correlate with occurrence in biology.

5.2.2 Introduction

Non-Watson-Crick motifs are highly prevalent among biological RNAs, and consist of a variety of structures including bulges (one or more nucleotides are unpaired on one strand, i.e. [1x0], [2x0], etc.), internal loops (one or more nucleotides unpaired in both strands, i.e. [1x2], [2x2], etc.), and mismatches ([1x1] internal loop). Mismatches in RNA provide backbone flexibility that can stabilize complex tertiary structures essential for functional RNA, as well as promote specific recognition of proteins through distortion of the major groove and exposure of alternative hydrogen bonding groups within the bases. In addition to single isolated mismatches [1x1], symmetric tandem mismatches are a common RNA motif [2x2] \( \text{A-A} \). Given the array of possible tandem mismatches, their disparate effects on the A-form structure of dsRNA, and their differential prevalence in biological RNAs, tandem mismatches present a possible target for recognition by RNA sensors in the innate immune response, such as PKR. Indeed, PKR is known to recognize RNAs with a variety non-Watson-Crick motifs via its tandem non-sequence-specific double-stranded RNA binding motifs (dsRBMs).

The tandem GA mismatch is an intriguing candidate for PKR regulation because it can adopt two distinct hydrogen-bonding patterns depending upon the identity of the base pairs immediately upstream and downstream of the mismatch. When flanked by GC
closing base pairs, each of the tandem mismatches adopts an imino conformation, in
which G–O6 and G–NH1 (imino) pair with A–NH6 and A–N1, respectively (Figure
5.2.1, left). Both of these pairing occur on the Watson-Crick faces, which categorizes the
imino conformation as “non-wobble”. When flanked by CG closing base pairs, each
tandem GA mismatch adopts a sheared conformation, in which G–NH2 and G–N3 on the
minor groove edge of the base pair with A–N7 and A–NH6 on the Hoogsteen face,
respectively (Figure 5.2.1, right).

---

**Figure 5.2.1** Hydrogen bonding within the two possible conformations of tandem GA
mismatches: imino (left) and sheared (right). Respective closing basepairs flanking each
mismatch conformation are depicted below the structures.

---

Due to the differences in basepairing geometry between the imino and sheared
conformations of tandem GA mismatches, the consequences on the shape of the major
groove near the mismatch are also different. Specifically, the tandem imino GA motif widens the surrounding narrow major groove due to the increased size of a Watson-Crick-face to Watson-Crick face purine-purine base pair relative to a purine-pyrimidine base pair, while maintaining the A-form geometry of the flanking backbone (Figure 5.2.2.A to C, left structures). In contrast, while the tandem sheared GA motif also retains an overall A-form geometry, the surrounding narrow major groove is narrowed due to the decreased inter-strand distance of the sheared conformation (Figure 5.2.2.A to C, right structures). In addition to determining the shape of the major groove near the mismatch, the base pairs flanking the tandem GA mismatch also determine its thermodynamic stability, such that the imino GA is ~2 kcal/mol more stable than the sheared.

There is a large disparity in the occurrence of these motifs in nature. The tandem imino GA motif, which is thermodynamically more stable than the sheared, is biologically absent, while the sheared GA motif is phylogenetically recurrent. Indeed, ~4% out of ~1000 tandem mismatches in a database of naturally occurring RNAs were sheared GA mismatches, the third most represented of all possible tandem mismatches, while no imino mismatches were identified. It has been previously shown that dsRNAs containing tandem AG mismatches, which are biologically rare and also adopt a non-wobble imino geometry, are able to activate PKR. Conversely, insertion of GU mismatches, which form wobble base pairs and are nearly omnipresent in functional RNAs, abrogates PKR activation. We thus hypothesized that of the two possible geometries of tandem GA mismatches, the sheared conformation predominates in nature due to immune-based selective pressure rather than thermodynamic bias: Specifically,
rare imino tandem GA mismatches may regulate PKR, whereas common sheared tandem GA mismatches may not.

We investigated this hypothesis through PKR activation and binding assays in the context of 33 bp dsRNA, 40 bp dsRNA, and a 16 bp RNA hairpin, containing one or more tandem mismatches. Through this investigation, we sought to determine if the tandem GA mismatch serves as RNA PAMP whereby PKR can distinguish between self-RNAs, which contain only the sheared mismatches, and non-self RNAs containing imino GA mismatches.
Figure 5.2.2 Solution NMR structures of model RNA duplexes containing imino (left structures, PDB ID: 1MIS\textsuperscript{29}) and sheared (right structures, PDB ID: 1YFV\textsuperscript{28}) GA mismatches. Duplexes are depicted from the major grooves in (A) stick and (B) space-filling representations. (C) Stick representations of respective hydrogen bonded mismatches and sequences of model duplexes depicted in (A) and (B).
5.2.3 Materials and Methods

5.2.3.1 RNA Sequences

The 79 bp (dsRNA-79) control was prepared by transcribing opposing strands of pUC19 derived PCR products and annealing as previously described. The remaining RNAs were prepared by \textit{in vitro} transcription from a hemi-duplex template (see below).

Following are the sequences of RNA used in this study.

**33 bp dsRNAs** Top strand (TS) and bottom strand (BS) sequences were annealed. Positions of GA mismatches and flanking base pairs are underlined. BS sequences contain two additional G’s at the 5’-end relative to TS sequences for priming transcription, resulting in a two nucleotide 5’-overhang in the resultant dsRNA. “I” and “S” in the name refers to imino and sheared.

33 bp-I-GA-TS: 5’GGAGGGUUCGCCUGGGACUCGGUCUGCUUGUUC
33 bp-I-GA-BS: 5’GGGAACAAGCAGACCCAGGGACAGCGCAGACCCUCC
33 bp-S-GA-TS: 5’GGAGGGUUCGCCUGGGACUGCCUGCUUGUUC
33 bp-S-GA-BS: 5’GGGAACAAGCAGACCGAGCGCAGCGCAGACCCUCC

**dsRNA16 hairpin RNAs** Loop nucleotides are in boldface and contain the U1A protein binding site, as these RNAs originated from PKR crystallography constructs designed by Laurie Heinicke. Positions of GA mismatches with flanking base pairs and corresponding Watson-Crick base pairs in WT sequence are underlined.

dsRNA16-WT:
5’GGAGAUACGAGACUGAUUGCACUCCCAUCUGCGUACUCUGCAGACUGACUGCAGACUGACUGCACUC

dsRNA16-I-GA:
5’GGAGAUGGACAGACUGAUUGCACUCCCAUCUGCGAUCUCUGCAGACUGACUGCAGACUGACUC

dsRNA16-S-GA:
5’GGAGAUGGAGACUGAUUGCACUCCCAUCUGCGAUCUCUGCAGACUGACUGCAGACUC

**40 bp dsRNAs** Top strand (TS) and bottom strand (BS) sequences were annealed. Positions of GA mismatches and flanking base pairs and corresponding Watson-Crick base pairs in WT sequence are underlined.

40 bp-WT-TS:
5’GGACCUGUGCGUGAUCGCCUGGAGCAUCUCUGUACGUCC
40 bp-WT-BS:
5’GGACGUAACAGGAUGCUCCAGGGAUCACGCACAGGUCC
40 bp-3X-I-GA-TS:
5’GGACCGUGGGACAUCCCUUGGACCAUCUCCUGGACACGUCC
40 bp-3X-I-GA-BS:
5’GGACGUAGGACAGGAUGGGACAGGAUGGACAGCACAGGUCC
40 bp-3X-S-GA-TS:
5’GGACUGUGCGAGAUCCUCAGCAUCUCAGAGUACGUCC
40 bp-3X-S-GA-BS:
5’GGACGUACGAGGAUGCGAGAGGAUCGAGCACAGGUCC

5.2.3.2 RNA preparation

Top and bottom strands of 33 bp and 40 bp dsRNAs were prepared by transcription from a hemi-duplex DNA template (IDT) with a T7 promoter. 0.25 µM hemi-duplex template was combined with T7 RNA polymerase, along with 40 mM Tris (pH 8), 25 mM MgCl₂, 2 mM DTT, 1 mM spermidine, and 3 mM of each NTP, and incubating at 37 ºC. After 4.5 h, reactions were quenched by adding 95% (v/v) formamide loading buffer. Hairpin RNAs (dsRNA16) were transcribed from synthetic dsDNA templates containing a T7 promoter (IDT) using T7 kits (Ambion). After incubating at 37 ºC for 6 h, reactions were quenched by adding formamide loading buffer. All RNAs were purified by fractionating on a polyacrylamide denaturing gel (7 M urea, 1X TBE). Transcripts were identified by UV shadowing, excised from the gel, and eluted overnight at 4 ºC in 1X TEN250. RNA was then ethanol precipitated and resuspended in 1X TE buffer (pH 7.5) and stored at -20 ºC. RNA concentration was determined spectrophotometrically.
5.2.3.3 Protein preparation

Full-length PKR and the dsRBD (p20) containing N-terminal His$_6$ tags were purified from *Escherichia coli* BL21(DE3) Rosetta cells (Novagen) as described previously.$^{5,19}$ The concentration of proteins was determined spectrophotometrically.$^{20}$

5.2.3.4 PKR activation and inhibition assays

RNAs were tested for their ability to activate or inhibit PKR autophosphorylation. PKR was first dephosphorylated by treatment with $\lambda$-PPase (NEB) for 1 h at 30°C, followed by the addition of the freshly made phosphatase inhibitor, sodium orthovanadate.$^{21}$ Next, 10 µCi [$\gamma$-$^{32}$P]ATP (Perkin-Elmer), 0.6 µM dephosphorylated PKR and RNA were incubated in 20 mM HEPES (pH 7.5), 4 mM MgCl$_2$, 100 mM KCl, and 100 µM ATP (Ambion) for 10 min at 30°C. In PKR inhibition studies, 79 bp dsRNA or 40 bp dsRNA were used as an activator at a constant concentration of 0.1 µM in the presence of various concentrations of inhibitor RNA. All reactions were quenched by adding SDS loading buffer. Samples were heated at 95°C for 5 min then loaded on 10% SDS-PAGE gel (Pierce). After electrophoresis, the gels were exposed to a storage PhosphorImager screen and the intensities of the labeled bands were quantified on a PhosphorImager (Molecular Dynamics). A background value was averaged from different portions of the gel and subtracted from each band prior to normalization.
5.2.3.5 Native mobility gel-shift assays

To determine the binding affinity of the dsRBD of PKR (p20) for dsRNA, gel-shift assays were conducted by first 5’-end-labeling 16 bp hairpin RNAs with $^{32}$P. RNAs were treated with CIP to remove the 5’-triphosphate and then kinased using PNK (NEB). RNAs were again purified by gel electrophoresis, excised, eluted overnight at 4 ºC, and ethanol precipitated. After resuspending in 1X TE, the concentration of 5’-end-labeled RNA was determined using a liquid scintillation counter (Beckman). To perform gel-shift assays, excess p20 (0.05-5 µM) was incubated with trace amounts of 5’-$^{32}$P-end-labeled RNA (~ 2 nM), 0.1 µg/µL yeast tRNA$^{Phe}$, 10 mM NaCl, 25 mM HEPES (pH 7.5), 5 mM DTT, 0.1 mM EDTA, and 5% glycerol for 30 min at 22 ºC. Samples were loaded onto 0.5X TBE native gels (29:1 crosslink) at 16 ºC while the gel was running, and fractionated for 2-2.5 h. Gels were dried and exposed to storage PhosphorImager screens overnight.

5.2.4 Results and Discussion

5.2.4.1 Effect of imino and sheared tandem GA mismatches in 33 bp dsRNA on PKR activation and inhibition

It has been established that PKR activation requires a minimum of 33 bp of RNA, with a bell-shaped dependence on RNA concentration, in which the RNA is recognized in a non-sequence specific fashion by the two tandem double-stranded RNA binding motifs (dsRBMs), which promotes PKR dimerization and activation. Additionally, 33 bp RNA is a potent inhibitor of 79 bp dsRNA-dependent PKR activation. Thus, 33 bp
RNA presents a convenient model system for investigating the impact of tandem GA mismatches on PKR activation. The minimal nature of 33 bp RNA constructs may “force” PKR to interact directly with the mismatch, rendering their ability to activate PKR particularly sensitive to imperfections in secondary structure. Additionally, effects of RNA binding to PKR can also be indirectly monitored through inhibition assays in which 33 bp RNA is titrated in increasing concentrations in the presence of a potent activator, 79 bp dsRNA. In this type of assay, the PKR–activating dimer is titrated into inactive monomers onto the shorter RNA. In summary, because 33 bp is the lower limit in length for activating PKR, it can function as both an activator and as a strong inhibitor in the presence of a stronger activating RNA.

We prepared two 33 bp dsRNAs with tandem GA mismatches located in the middle of the duplex (Figure 5.2.3.A): one containing a tandem imino GA (33 bp-I-GA), the other containing a tandem sheared GA (33 bp-S-GA). Both RNAs were tested for PKR activation by incubating with dephosphorylated PKR over a range of RNA concentrations (0.15-5 μM). Inhibition of 79 bp-dependent PKR activation was also tested by incubating 0.1 μM 79 bp RNA with increasing concentrations (0.15-5 μM) of 33 bp-I-GA or 33 bp-S-GA (Figure 5.2.3.B). Both imino and sheared tandem GA-containing RNAs weakly activated PKR, although the degree of activation was essentially the same for both RNAs (Figure 5.3.B, left hand lanes). This activation is somewhat surprising because perfect 33 bp RNA is a very weak activator, as shown previously,19 and introduction of mismatches effectively decreases the total number of canonical base pairs available for PKR binding. PKR may tolerate these bulges, however, due to their symmetrical nature, as it has been proposed that symmetric non-
Watson-Crick motifs in RNA may contribute to PKR activation due to their overall A-form helical geometry.\textsuperscript{13}

\textbf{Figure 5.2.3} Activation and inhibition of PKR by 33 bp dsRNA containing one centrally positioned tandem GA mismatch. (A) Sequence of 33 bp dsRNAs containing imino (33 bp-I-GA) and sheared (33 bp-S-GA) GA mismatches. Positions of mismatches and adjacent base pairs are boxed. (B) PKR activation (left sets of lanes) and inhibition (right sets of lanes) assays of 33 bp-I-GA (top gel) and 33 bp-S-GA (bottom gel). RNA concentrations for both assays in both gels were 0.15, 0.31, 0.63, 1.25, 2.5, and 5 µM. For inhibition assays, the concentration of 79 bp RNA in all lanes was 0.1 µM. For all (B), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. A no-RNA lane and a no-inhibitor-RNA lane are included. Phosphorylation activities are noted under the gels and are normalized to the 79 bp no-inhibitor-RNA lane in the top gel. Fold-effects are provided. (C) Graphical representation of phosphorylation activities from panel (B) as a function of RNA concentration.
In the case of PKR inhibition assays, the RNA with the tandem imino-GA (33 bp-I-GA) was a slightly better inhibitor than sheared-GA across the range of RNA concentrations tested (up to 1.6-fold better inhibition [Figure 5.3.B, right hand lanes]). It was expected that a 33 bp RNA containing a tandem imino GA mismatch would be a better regulator of PKR because the rarity of imino versus sheared GA mismatches in biology. While 33 bp-I-GA did inhibit PKR to a greater degree than 33 bp-S-GA, the effect was minimal and extent of inhibition for both RNAs was similar to published values for wild-type 33 bp RNA. This suggests that PKR may bind 33 bp RNA in more than one register such that it can evade contact with the mismatch.

5.2.4.2 Binding and inhibition of PKR by 16 bp RNA hairpins containing imino and sheared tandem GA mismatches

Within the RNA binding domain of PKR, the two tandem dsRBMs are connected by a flexible linker. Biochemical studies have shown that both dsRBMs of one PKR monomer can bind a 16 bp segment of RNA, with each dsRBM spanning 11 bp of the RNA, as depicted in Figure 5.2.4. Because of the connecting flexible linker, each dsRBM is able to bind a different face of the RNA, allowing for possible overlapping binding of the dsRBMs on the same RNA. According to this model, a 16 bp hairpin RNA meets the minimal requirements for binding one PKR monomer. Due to this minimal nature, the insertion of a tandem GA mismatch into 16 bp hairpin RNA should eliminate the possibility of PKR evading contact with the mismatch through binding of alternative registers, thus providing an effective model for screening the effect of imino versus sheared GA mismatches on PKR inhibition.
Figure 5.2.4 Overlapping binding of dsRBM 1 and dsRBM 2 on a single dsRNA.

Three 16 bp hairpin RNAs were transcribed, one containing a tandem imino GA mismatch at the midpoint of the hairpin stem (dsRNA16-I-GA), one containing a tandem sheared GA mismatch at the same position (dsRNA16-S-GA), and another containing all Watson-Crick basepairs in the stem (dsRNA16-WT) (Figure 5.2.5.A). Because these RNAs represent the lower limit of duplex length for PKR binding, native mobility gel-shift assays with each RNA and the dsRBD of PKR (p20) were first performed to ensure that insertion of non-Watson-Crick elements into stem portion of the hairpin loop does not complete abrogate PKR binding.

Complex formation was observed for all three RNAs, starting at 1.25 µM p20 for dsRNA16-WT and dsRNA16-S-GA, and 0.5 µM p20 for dsRNA16-I-GA (Figure 5.2.5.A). In addition to displaying complex formation at a lower p20 concentration, the fraction bound of dsRNA16 containing the imino tandem GA was 2.5-fold higher than for the WT or sheared GA forms (Figure 5.2.5.B). Also, the faster migration of the WT version of the hairpin versus the RNAs containing mismatches is consistent with the more compact nature of the perfect duplex versus duplexes containing bulges and imperfections. This binding assay indicates PKR tolerates local distortions to the major
Figure 5.2.5 Native mobility gel-shift assays of p20 binding to RNA hairpins containing tandem GA mismatches. (A) PKR dsRBD (p20) binding to dsRNA16 containing no mismatch (dsRNA16-WT, left panel), an imino GA mismatch (dsRNA16-I-GA, center panel), and a sheared GA mismatch (dsRNA16-S-GA, right panel). Secondary structures of RNAs with positions of mismatches and adjacent base pairs (and corresponding position in WT sequence) boxed, are depicted above each respective panel. For all panels, trace amounts of radiolabeled RNA were incubated with p20 at the following concentrations: 0.05, 0.125, 0.25, 0.5, 1.25, 2.5, and 5 μM. Samples were analyzed by 10% native PAGE. Positions of unbound and complexed RNA are indicated. (C) Graphical representation of gel-shift assay depicted in (A). The fraction bound for each RNA is plotted as a function of p20 concentration.

We next performed PKR activation and inhibition assays on dsRNA16-WT, dsRNA16-I-GA, and dsRNA16-S-GA. Over the range of RNA concentrations tested (0.15-5 μM), PKR was not significantly activated by any of the three hairpin RNAs, as expected, given that activation requires ~33 bp of RNA (Figure 5.2.6.B and C, left hand lanes). Inhibition of 79 bp-dependent PKR activation was also tested by incubating...
Figure 5.2.6 Activation and inhibition of PKR by 16 bp dsRNA hairpins containing a tandem GA mismatch. (A) Secondary structures of hairpins depicted in Figure 5.2.5.A. Positions of mismatches and adjacent base pairs, and corresponding position in WT sequence, are boxed. (B) PKR activation (left sets of lanes) and inhibition (right sets of lanes) assays of dsRNA16-WT (top gel), dsRNA16-I-GA (middle gel), and dsRNA16-S-GA (bottom gel). RNA concentrations for both assays in all gels were 0.15, 0.31, 0.63, 1.25, 2.5, and 5 µM. For inhibition assays, the concentration of 79 bp RNA in all lanes was 0.1 µM. For all (B), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. A no-RNA lane and a no-inhibitor-RNA lane are included. Phosphorylation activities are noted under the gels and are normalized to the 79 bp no-inhibitor-RNA lane in the bottom gel. Fold-effects are provided. (C) Graphical representation of phosphorylation activities from panel (B) as a function of RNA concentration.
increasing concentrations of each RNA (0.15-5 µM) with 0.1 µM 79 bp RNA (Figure 5.2.6.B and C). The degree of inhibition for all three of the RNAs, however, was very slight (1.5- to 1.9-fold) relative to inhibition by the 33 bp RNAs (24- to 30-fold). Given the fact that 79 bp RNA is a very strong activator of PKR, it is likely that the 16 bp hairpin RNAs, which contain the shortest number of basepairs possible to bind a PKR monomer, cannot effectively compete in the presence of such a robust activator.

We thus repeated the inhibition assay in the presence of a weaker PKR activator, 40 bp RNA (0.1 µM), over the same range of hairpin RNA concentrations (Figure 5.2.7.A and B). In this case, RNA containing the tandem imino GA (dsRNA16-I-GA) was again a more effective inhibitor relative to RNA containing the sheared GA mismatch (3.8-fold versus 2-fold), as well as wild-type RNA (2.6-fold). These data thus suggest that in the context of a short inhibitory RNA, PKR may be preferentially regulated, albeit slightly, when a tandem GA mismatch has an imino geometry rather than sheared geometry or Watson-Crick.

5.2.4.3 Effect of multiple tandem GA mismatches on PKR activation by 40 bp RNA

Thus far the effect of isolated tandem GA mismatches have been investigated, but it is possible that tandem mismatches can exist in multiple registers within the same RNA. Given that adding multiple tandem mismatches in RNA leads to a further decrease in stability relative to a single tandem mismatch, the effects of additional mismatches on PKR activation could be cumulative. Thus, 40 bp duplex RNAs containing three sets of imino (40bp-3X-I-GA) and sheared (40bp-3X-S-GA) tandem GA mismatches were prepared (Figure 5.2.8.A).
Figure 5.2.7 Inhibition of PKR activation by 16 bp dsRNA hairpins containing a tandem GA mismatch in the presence of a 40 bp dsRNA activator. (A) PKR activation and inhibition assays. No-RNA and 79 bp control lanes are included. Activation assays (left sets of lanes) of dsRNA16-WT (top gel), dsRNA16-I-GA (middle gel), and dsRNA16-S-GA (bottom gel) were performed as in Figure 5.2.5.B. Phosphorylation activities are noted under the gels and are normalized to the 79 bp lane in the middle gel. For inhibition assays (right sets of lanes), the concentration of 40 bp dsRNA was 0.1 µM in all lanes, and inhibitor RNA concentrations were 0.15, 0.31, 0.63, 1.25, 2.5, and 5 µM. Phosphorylation activities (boldface) are noted under the gels and are normalized to the lanes in which only 40 bp RNA was present. Fold-effects are provided. 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. (B) Graphical representation of phosphorylation activities from (A) as a function of RNA concentration.

The wild-type sequence of this RNA (40bp-WT), which does not contain any mismatches or other helical imperfections, was based on model RNAs used to investigate the effect of bulge size and position on eletrophoretic mobility\(^{44}\) and PKR activation\(^{46}\), and was designed to form a stable duplex with little self-structure in the individual
strands. The three tandem mismatches were placed within this RNA with 6-7 bp in between each set and 7-9 bp from either end of the RNA to minimize effects of end fraying.

As with the previously discussed 33 bp and hairpin RNAs, we performed PKR activation and inhibition assays on 40bp-3X-I-GA, 40bp-3X-S-GA, and 40bp-WT. (Figure 5.2.8.B) At the lowest RNA concentration tested (0.15), 40bp-WT activated PKR up to 27% the level of activation by 79 bp RNA, which falls in the inhibitory arm of the bell-shaped dependence of PKR activation on RNA concentration, as activation decreased over the remaining RNA concentrations tested (Figure 5.2.8.B and C). Neither 40bp-3X-I-GA nor 40bp-3X-S-GA were effective activators of PKR, indicating that 40bp RNA, which is a relatively weak activator, cannot tolerate this degree of sheared or imino helical imperfection (Figure 5.2.8.B and C). This notion is consistent with the fact that upon introduction of three sets of tandem mismatches, the effective number of base pairs is reduced to 34, which is essentially the minimal number possible for activation. Additionally, the relatively close spacing of the mismatches within the RNAs results in only 9 bp or fewer in succession. This mismatch configuration likely prevents PKR from avoiding contact with these imperfections and results in the loss of activation relative to wild-type.

Inhibition assays with 40 bp RNAs containing three sets of tandem mismatches and wild-type 40 bp RNA were also performed. Similar to the results observed for 33 bp RNA, 40 bp-WT serves as a PKR activator as well as a potent inhibitor, exhibiting up to 26-fold inhibition (Figure 5.2.8.B and C). The 40 bp RNAs containing multiple tandem mismatches were also effective inhibitors. In this context, RNA containing three imino
GA mismatches (40 bp-3X-I-GA) was a stronger inhibitor, with up to 33.3-fold inhibition, compared to the sheared GA version (40 bp-3X-S-GA) which displayed only 9.5-fold inhibition. Although the maximum extent of PKR inhibition by 40 bp-3X-I-GA was slightly greater than for 40 bp-WT, which occurs for both at 5 µM inhibitor RNA, at all other RNA concentrations tested, inhibition was greater for wild-type. Relative to 40 bp-3X-S-GA, however, inhibition by 40 bp-3X-I-GA was 1.2- to 3.5-fold more potent at every RNA concentration tested, suggesting that in the context of an inhibitor RNA containing multiple tandem GA mismatches, PKR is again regulated preferentially by mismatches possessing imino geometries over sheared, with a greater extent of preference relative to a single tandem mismatch (3.5-fold greater for multiple mismatches versus 1.9-fold for a single mismatch).
Figure 5.2.8  Activation and inhibition of PKR by 40 bp dsRNA containing three sets of tandem GA mismatches.  (A) Sequence of 40 bp dsRNAs containing no mismatch (40 bp-WT), three imino GA mismatches (40 bp-I-GA), and three sheared GA mismatches (40 bp-S-GA).  Positions of mismatches and adjacent base pairs and corresponding position in WT sequence are boxed.  (B) PKR activation (left sets of lanes) and inhibition (right sets of lanes) assays of 40 b-WT (top gel), 40 bp-3XI-GA (middle gel), and 40 bp-3XS-GA (bottom gel).  RNA concentrations for both assays in all gels were 0.15, 0.31, 0.63, 1.25, 2.5, and 5 µM.  For inhibition assays, the concentration of 79 bp RNA in all lanes was 0.1 µM.  For all (B), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated.  A no-RNA lane and a no-inhibitor-RNA lane are included.  Phosphorylation activities are noted under the gels and are normalized to the 79 bp no-inhibitor-RNA lane in the top gel.  Fold-effects are provided. (C) Graphical representation of phosphorylation activities from panel (B) as a function of RNA concentration.
5.2.5 Conclusions

Traditionally, PKR was strictly thought of as the “double-stranded RNA” protein kinase that functioned in innate immunity through recognition of long RNA helices. More recently, however, several studies have shown that PKR is regulated by RNAs with various structural motifs and helical imperfections, which has revealed the rich complexity of PKR activation by RNA. As a consequence, a detailed set of rules for predicting RNA activators and inhibitors of PKR is desired. Part of this is determination of the characteristics that enable PKR to recognize one set of motifs as hallmarks of pathogenic RNA that would trigger regulation while identifying other motifs as belonging to cellular RNA. In an effort to increase our understanding of these rules, we investigated the possible role of tandem GA mismatches in PKR regulation. Upon slight differences in adjacent basepairs, the GA mismatch can adopt two distinct geometries with contrasting representation in biological RNAs. The structural and phylogenetic disparity between these two types of tandem GA mismatches suggested that they might serve as targets for PKR discrimination between self and non-self RNA.

We studied the tandem GA motif in the context of three RNAs, as summarized in Table 5.2.1. We predicted that PKR would be regulated most potently by RNA containing the rare tandem imino GA mismatch, which could serve as a recognition motif of non-self RNA for PKR. Across all three sets of RNAs studied, PKR inhibition was slightly favored for RNAs containing imino versus sheared tandem mismatch. Adding additional mismatches to the same RNA increased the fold-effects from ~1.5-2-fold up to 3.5-fold. These effects are consistent with expectations, although the effects are modest.
Table 5.2.1 Summary of PKR inhibition results. Values for “Max PKR Inhibition” represent fold inhibition of 0.1 µM 79 bp RNA for 33 bp-\textit{Y}-GA and 40 bp-3X-\textit{Y}-GA, and inhibition of 0.1 µM 40 bp RNA for dsRNA16-\textit{Y}-GA.

<table>
<thead>
<tr>
<th>RNA (where ( Y = I ) or ( S ))</th>
<th>Max PKR Inhibition</th>
<th>fold-effect ( I ) over WT</th>
<th>fold-effect ( I ) over ( S )</th>
</tr>
</thead>
<tbody>
<tr>
<td>33 bp-\textit{Y}-GA</td>
<td>31</td>
<td>24</td>
<td>–</td>
</tr>
<tr>
<td>dsRNA16-\textit{Y}-GA</td>
<td>3.8</td>
<td>2</td>
<td>2.6</td>
</tr>
<tr>
<td>40 bp-3X-\textit{Y}-GA</td>
<td>33.3</td>
<td>9.5</td>
<td>26</td>
</tr>
</tbody>
</table>

In light of these results and the existing phylogenetic characterization of the two forms of tandem GA mismatches, possible future experiments would involve identifying known biological RNAs, such as miRNA precursors, that contain sheared GA mismatches but do not regulate PKR. Changing the flanking base pairs of these RNAs to convert the tandem mismatches to the imino form might transform the RNAs into PKR regulators. Such results could have implications not only for predicting biological RNA regulators of PKR but also for understanding protein-RNA interactions of other dsRBD-containing proteins, such as Dicer and Drosha.
5.3 References


VITA

Rebecca Toroney

Education
Ph.D., Chemistry, The Pennsylvania State University, December 2010
B.A., Chemistry, Franklin and Marshall College, May 2004

Publications


Selected Honors
Department of Chemistry Geiger Fellowship (Graduate Student Research Award), The Pennsylvania State University, 2010
Best Thematic Poster Award, ASBMB Annual Meeting, 2010
Department of Chemistry Norma Robinson Scholarship (Graduate Student Research Award), The Pennsylvania State University, 2008-2009
Department of Chemistry Graduate Student Leadership and Service Award, The Pennsylvania State University, 2007
Department of Chemistry Roberts Fellowship, The Pennsylvania State University, 2004-2005